

# PROCESS SCALE PURIFICATION OF ANTIBODIES



EDITED BY  
Uwe Gottschalk

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Edited by

**UWE GOTTSCHALK**

Group Vice President  
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# PREFACE

Monoclonal antibodies are an important component of the biopharmaceutical industry. Within the burgeoning market of protein-based therapeutics, they are the market leaders in terms of volume sales and the most common class of product. Almost all commercial antibodies are produced in cultured mammalian cells, and an entire subindustry has grown up around downstream processing to ensure that manufacturing processes generate safe and pure products suitable for administration to humans. This is an industry in which I have been involved for many years, and one that is facing exciting and difficult challenges.

I first came into contact with the world of monoclonal antibodies in 1986, when their production in cultured mammalian cells was still in its infancy. This was at the Cancer Research Campaign Laboratories in Nottingham, UK. I was a PhD student from Germany, and one of my main tasks was to purify antibodies from mouse ascites, a horrible process for obvious reasons. A milligram of antibodies was worth far, far more than its weight in gold.

At the time, my colleagues and I had visions of curing cancer by drug targeting, and we linked all sorts of cytotoxic agents to the antibodies we produced. Unfortunately, some of the expectations surrounding the medical use of antibodies turned out to be premature and unrealistic. Our awareness of this coincided with the first real downturn in the biotechnology sector, but antibodies survived in niche markets for diagnostics and research reagents. Years later, new life has been breathed into therapeutic antibodies and they are now back, stronger than ever. Indeed, they represent the fastest-growing area in biotherapeutics with 21 products on the US market (as of 2007) and hundreds in clinical and preclinical development (1).

At the end of the 1980s, antibodies were produced commercially using mammalian cells cultured in perfusion fermenters, but yields rarely exceeded



100mg/L. Huge volumes of culture broth needed to be processed, and the easiest way to bring the volume down was polyethylene glycol (PEG) precipitation with tons of material and endless centrifugation cycles. The yields were poor and difficult to reproduce, but there were no alternatives. Since that time, the productivity of cell cultures has increased significantly, with 1–5g/L titers now routine and the real prospect of 10–20g/L yields in the next decade. How far we have come since the early days of biomanufacturing!

The increase in titers has heaped pressure on the downstream processes that we use to extract and purify antibodies from cell culture broth, and the technologies used in downstream processing have been forced to modernize and improve in the face of this increasing challenge. There is little doubt that packed-bed chromatography is the workhorse of current downstream processing, its high resolution and relative simplicity making it the central enabling technology in modern bioseparation processes (2). As productivity increases, however, doubts have been cast on the ability of column chromatography to cope with the dramatically increasing product titers in fermentation (3). Unlike fermentation, capturing steps in downstream processing have hardly any economy of scale. Bind-and-elute cycles in chromatography are driven by mass rather than by volume, and this means that increasing batch sizes translate into increasing costs in a near linear fashion. This phenomenon particularly affects the first column, where all of the product must be captured. This initial recovery step has therefore been identified as the most serious potential bottleneck, with knock-on effects throughout the processing facility, e.g., in terms of column sizes, buffer preparation, and hold. However, this opinion is not shared by everyone, and debate continues as to whether or not packed-bed chromatography is here to stay (4, 5).

These challenges and their surrounding issues set the scene for this exciting book, in which I have compiled a selection of chapters from top-tier industrial and academic experts providing up-to-date accounts of current best practice in the manufacture of monoclonal antibodies. Opinions on the suitability of packed-bed chromatography in today's manufacturing environment differ, particularly in the light of emerging competitive technologies, and the first chapter by Ann Lee and colleagues captures that debate and puts the case for and against the continuing reliance on traditional chromatography methods. The second chapter by John Curling provides an informative historical overview of the development of antibody purification technologies, providing the basis for the next five chapters, which consider some of today's major steps in antibody processing—harvesting and recovery (Abhinav Shukla and colleagues), Protein A chromatography (Suresh Vunnum and colleagues), non-Protein A strategies (Alahari Arunakumari and Jue Wang), mixed mode chromatography (Pete Gagnon), and integrated polishing (Sanchayita Ghose and colleagues).

Looking closer, the pace at which fermentation is guiding the way is not the only challenge for modern downstream processing. The regulatory frame-

work, particularly current good manufacturing practice (cGMP) is a moving target, and quality requirements are constantly leading to tighter specifications and higher safety margins, e.g., with regard to small, nonenveloped viruses. The chapter by Joe Zhou therefore deals with orthogonal methods for virus removal, before we consider platform technologies that integrate virus clearance with capture and purification (Yuling Li and colleagues). Nuno Fontes and Robert van Reis then consider the important aspects of scaling up antibody purification to industrial levels with a platform of methods that offer the potential to set a new standard in antibody manufacture. Finally in this section, Thomas Müller-Späth and Massimo Morbidelli consider the use of continuous chromatography for the high-resolution separation of antibodies, based on a laboratory-scale strategy they developed.

The next two chapters look at the economic perspectives of antibody manufacture, one from the standpoint of process economics (Suzanne Farid) and the other from the standpoint of process design and optimization (Andrew Sinclair). We then turn to the consideration of emerging technologies, which may replace, augment, or supplement traditional chromatography: flocculation, precipitation, and membrane adsorbers for antibody purification (Jörg Thömmes and Uwe Gottschalk); precipitation for the elimination of impurities (Judy Glynn); and charged filtration membranes (Mark Etzel).

While most of the book focuses on the purification of typical, full-size IgG molecules produced in fermenters, the final section deals with noncanonical antibody varieties and novel sources. There are chapters dealing with the purification of antibody fragments (Mariangela Spitali) and non-IgG monoclonals (IgM and IgA; Charlotte Cabanne and Xavier Santarelli), followed by a chapter considering the promising use of plant-based systems for antibody manufacture, and the particular challenges faced when isolating antibodies and other biopharmaceuticals from plant sources (Zivko Nikolov and colleagues).

The final chapter wraps up the book by looking to the future and considering what drives change in the industry, particularly what factors are likely to influence the techniques and technologies that will be adopted for antibody purification in the decade to come. This concluding chapter is written by Hari Pujar, Duncan Low, and Rhona O'Leary, three distinguished authors representing the top-tier companies in the sector.

In all likelihood, we will not see a revolution in downstream processing like the one that has galvanized upstream process development over the last 20 years. The chapters in this book are, however, evidence that the future of antibody purification holds great promise, underlining the progress that has been made in closing the performance gap between upstream production and downstream processing.

All the contributors to this book live and die for the production of antibodies. Some of us have been there from the very first day, while others have joined more recently, but we all passionately believe that technological advances and innovation can help to break through the current ceiling in

antibody processing and can lead to affordable, high-quality pharmaceutical products in the future.

UWE GOTTSCHALK

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## **DOWNSTREAM PROCESSING OF MONOCLONAL ANTIBODIES: CURRENT PRACTICES AND FUTURE OPPORTUNITIES**

BRIAN KELLEY, GREG BLANK, AND ANN LEE

### **1.1 INTRODUCTION**

Monoclonal antibodies (mAbs) are now established as the most prevalent class of recombinant protein therapeutics. They can be expressed at high levels in cell culture, are typically very soluble, and are relatively stable during processing. The nearly universal use of mammalian cell expression systems for mAb synthesis, combined with the selection of homologous, humanized mAb framework protein sequences, provides opportunities to harmonize manufacturing processes around base platforms that can then be used with only slight variations from product to product. In addition, by using a platform process, manufacturing plants designed for the production of one mAb can usually be readily adapted to produce others.

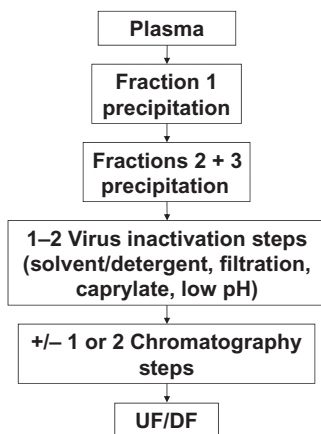
For these reasons, mAbs represent a unique group of biological products. They accommodate rapid process development time lines, can be produced in large quantities, and may be manufactured in multiple facilities during their lifecycle as a result of their common process flowsheets. As a result, they have relatively low manufacturing costs and benefit from the flexibility of production at either in-house or contract production facilities. Although mAbs are not commodity products that are substitutable in the clinical setting, they have

distinct advantages in production scale and cost, as well as in product development speed and convenience, when compared to other recombinant protein therapeutics.

This introductory chapter attempts to set the context for the following chapters, which cover many aspects of mAb purification in detail. A typical mAb purification process flow sheet is described and used to illustrate the impact of purification platforms on mAb production. Factors to consider with respect to the various process alternatives or new technologies described in upcoming chapters are addressed, emphasizing the integration of unit operations and process design principles into an optimized, holistic process. Both current practices and controversial topics are introduced, among them the challenges of very large-scale (VLS) production, issues related to facility fit, the maturation of process purification technology for mAb processing, the need for innovations in mAb downstream processing, and the impact of the evolving regulatory environment. It is hoped that this backdrop will stimulate critical thinking and comprehensive analysis when the processing options described in the following chapters are being considered.

## 1.2 A BRIEF HISTORY OF cGMP mAb AND INTRAVENOUS IMMUNOGLOBULIN (IgIV) PURIFICATION

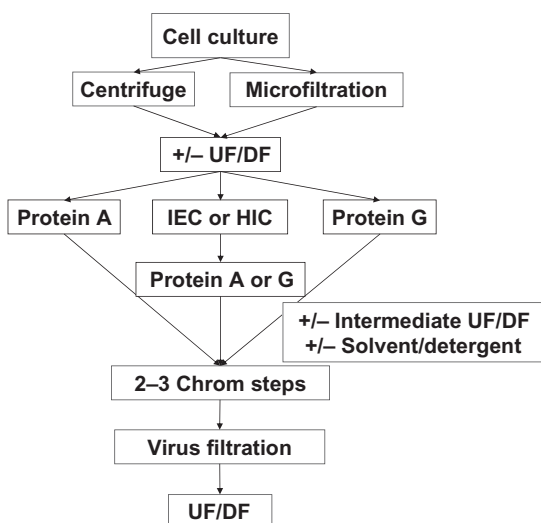
The processes used for production of IgIV from human plasma differ from those used for recombinant mAbs. Figure 1.1 shows a consensus processing scheme, based on many published process flow sheets, for the purification of IgIV. Most IgIV processes lack chromatographic steps and instead rely on multiple fractional precipitation steps based on the Cohn process developed in the 1950s (1). Some recently developed processes include chromatographic



**FIGURE 1.1** Cohn-based IgIV consensus process.

steps, but this is used to a limited extent and still in combination with upstream steps based on the Cohn process (2, 3). The processes used for recombinant mAb purification have borrowed very little from plasma fractionation technology, other than ultrafiltration to formulate and to concentrate the drug substance. The low cost of manufacturing IgIV and the very large production scale have led to debate on the value of going “back to the future” and applying IgIV processing technologies to the production of recombinant mAbs. A review of current mAb processing platforms will put this proposal into context.

The first cGMP for mAb purification reflected the state of the art in the 1980s and early 1990s, prior to the accumulation of substantial process knowledge and the introduction of improved separation media that made today’s more efficient and scalable processes possible. Examples of the diversity of early processes include the use of various microfiltration or depth-filtration media for harvest; affinity chromatography with Protein G in addition to Protein A; conventional capture columns to protect the Protein A resin; incorporation of challenging separation methods for large-scale production, such as size exclusion chromatography (SEC); solvent/detergent virus-inactivation methods; and the requirement for four or even more chromatography steps (Figure 1.2). In addition, downstream processing was sometimes performed in the cold. Chromatographic media often provided relatively low loading capacities, which were not a significant issue when cell culture titers were measured in hundreds of milligrams per liter. To address the need for kilogram-scale production, very large bioreactors were used; the focus for capture resin selection was based on maximizing volumetric productivity and on the ability to

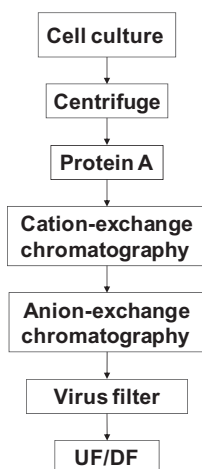


**FIGURE 1.2** Early mAb purification schemes. IEC = ion-exchange chromatography; HIC = hydrophobic interaction chromatography.

process large volumes of feed rapidly, rather than on the handling of large batches (greater than 20 kg of product). Many of these early mAb products were also derived from a more diverse set of framework protein sequences, reflecting the historical progression from murine and chimeric mAbs to today's fully humanized antibodies, which gave rise to a more varied set of process flow sheets.

### 1.3 CURRENT APPROACHES IN PURIFICATION PROCESS DEVELOPMENT: IMPACT OF PLATFORM PROCESSES

Despite the high degree of homology among humanized mAbs, variations in complementarity-determining regions and framework sequences make it difficult to define a truly generic purification process capable of processing many different mAbs without any changes to the operating conditions. Despite these variations, many companies have defined platform purification processes based on a common sequence of unit operations. A frequently used purification platform for mAbs is shown in Figure 1.3. The conditioned medium is first clarified by centrifugation, followed by depth filtration. Protein A chromatography offers direct product capture from the centrate and provides excellent purification and significant concentration of the product. The low-pH elution from the Protein A step also provides virus inactivation. Two chromatographic polishing steps are used to reduce host cell, medium, purification process-related impurities and product impurities. Additional virus removal is usually achieved in these polishing steps. One of the polishing steps is almost invariably anion-exchange (AEX) chromatography, often run in the flow-through mode. The second polishing step is typically cation-exchange (CEX) chromatography, although occasionally ceramic hydroxyapatite or hydrophobic inter-



**FIGURE 1.3** Typical mAb platform (current).

action chromatography (HIC) are used. The remaining process steps include virus filtration (VF) and ultrafiltration/diafiltration (UF/DF) to formulate and concentrate the product, which is now the bulk drug substance. The efficiency, robustness, and scalability of this standardized process have resulted in the rapid convergence of process development groups in the industry around a similar process flow sheet (4, 5).

The establishment of platform processes for mAb production has already had an enormous impact on process development strategy and activities, and is just beginning to affect the world of commercial manufacturing. At this point, very few companies have two or more commercial mAbs that are purified by a common platform process. Many mAbs currently in the clinical pipeline, however, are manufactured by a process similar to the standard process shown in Figure 1.3. The gradual progression of these early-stage processes from clinical to commercial production enables additional efficiencies in production that will reduce the costs of goods (COGs) and accelerate responses to surges in product demand. The benefits of efficient facility management (e.g., reductions in changeover time and the use of common raw materials and equipment) and flexible commercial production (e.g., balanced production schedules among multiproduct facilities) will be realized more slowly than the gains seen today with product candidates in the early stages of clinical development. The combination of platform processing, multiproduct facilities, rapid product changeover, and flexible sourcing between contract manufacturing organizations (CMOs) and in-house production facilities achieves an industrialization of mAb production that will be unprecedented in the field of recombinant protein biologics. Antibodies could become a class of therapeutic biologics that support the treatment of large patient populations while remaining cost competitive with small molecules. To achieve this vision, the biopharmaceutical industry must take advantage of the opportunities presented by the ease of development, validation, and production afforded by platform processes.

Given the value and broad adoption of processing platforms, combined with an installed production facility base designed for them, there is enormous pressure to conform to such platforms with future products. As a result, options for unit operations, raw materials, step sequences, control systems and algorithms, and processing equipment are limited. While these restrictions may at first seem highly constraining, they require other challenges to be addressed, e.g., the establishment of highly efficient work processes that rapidly define the appropriate processing conditions for each new mAb that enters the pipeline, as well as the definition of a common set of optimization approaches and process characterization studies that will streamline late-stage development of clinical products.

Clarification operations such as centrifugation often vary little from product to product, provided that the cell culture process is not radically different. Large changes in the cell concentration or viability in a bioreactor will affect clarification, but provided that the unit operations are designed for the worst-

case feedstream, few if any modifications will be needed for new mAbs. The capacity of centrate depth filters can vary significantly, depending on feedstream, and should be optimized for robustness while the costs of raw materials are minimized. Similarly, the platform's ultrafiltration steps (VF and final ultrafiltration) should be largely unaffected by the change in the mAb. The unit operations that are most likely to require tuning are the chromatography steps. Even there, the standardization of many elements in a chromatographic unit operation will streamline development time lines by focusing on key factors influenced by product characteristics (5). Process variables that are often specified for platform processes include resin and membrane selection, column bed height, wash volumes, loading capacity, membrane flux, and target bulk concentration. This effort simplifies and accelerates early-stage process development.

The Protein A capture step is generally a very robust operation that can tolerate changes in bioreactor harvest conditions and product characteristics (see Chapter 4). The variables that may be influenced by product or feedstream variations are dynamic binding capacity, the optimal composition of the column wash solution, and the elution conditions. Variations in these process parameters arise from differences in the affinity of Protein A for the mAb, the steric hindrance among molecules (6), and variations in impurity levels and species in the feedstream, probably caused either by the cell line and bioreactor management or by the properties of the mAb itself.

The most common variables for the ion-exchange polishing steps include the column-loading and solution compositions (e.g., pH and counterion concentration) and the wash and elution compositions. In some cases, there can be major changes to the platform, as when a highly acidic mAb has strong affinity for an AEX resin and the typical flow-through operation must be abandoned in favor of a bind-elute step. Broad ranges in the affinities of ion-exchange resins have been described (5, 7) and highlight one area where the diversity of mAb properties has an impact on the purification process.

Given that chromatography variables may be optimized independently for each mAb, there are choices to be made regarding the investment in early-stage process development. Two extremes are represented by tailored, as compared to generic, processing conditions. An example of the tailored approach is the use of high-throughput screening to define unique operating conditions for each mAb, e.g., to optimize an AEX polishing step (8). Similarly, scouting studies using gradient elution for bind-elute steps could be used to tune the elution conditions for a CEX step. The generic approach to process development would use a single pH for the AEX step and adjust the load counterion concentration only by dilution. This minimum counterion concentration would vary from mAb to mAb; an even more flexible step would use a single counterion concentration, which allows successful processing of the majority of mAbs without significant product losses of the most acidic family members. Likewise, in a generic approach for CEX, the step could be designed with a very low conductivity for the load combined with a broad gradient

elution, which could potentially encompass successful processing of a large number of mAbs (see Chapters 5–7, which provide examples of this approach). The trade-offs of the two approaches would be influenced by the interplay of process development resources and time (more for the tailored approach, less for the generic), manufacturing efficiency (higher for tailored, lower for generic), and other factors, such as mAb characteristics.

After Phase I process development, companies typically engage in at least one additional cycle of process development (commonly termed late-stage development), which defines the Phase III process and is subsequently used for product launch. This second cycle often involves changes to the cell culture process, including media reformulation, changes to the feeding strategy, optimization of culture duration, and potentially the introduction of a new cell bank or cell line. Although changes to the purification process during this second cycle are unlikely to have the same regulatory impact as cell line changes, the elimination of a step (e.g., the elimination of one of two polishing steps) could cause a problematic change in an impurity profile. Given that several years may have passed between the development of the Phase I and Phase III processes, consideration should be given to upgrading the purification process to include the superior separation media that have been recently introduced to the market. Additional optimization studies will define the final process control ranges for key and critical process parameters and will investigate the processing parameters that are unique to each mAb (e.g., column capacities, resin and membrane lifetimes, in-process hold times, and maximum bulk concentration).

If the implementation of radically new processing technologies is considered, this option is typically weighed at the late-stage development cycle rather than at the early stage. Alternate, off-platform technologies (9) would require sustained and significant effort to define processing parameters; establish process robustness; acquire and test novel raw materials; and specify, purchase, and validate new equipment. These factors strongly support an approach where implementation of new technology on a Phase I time line would be used only if the new process technology represented the sole means of enabling clinical production. The “speed-to-clinic” driver would almost always trump the benefits in COGs or the productivity benefits that novel technology would offer at this stage of development. This would force novel technologies to be considered during the next two cycles of process development, either for the Phase III definition or as a post-licensure change.

This argument may appear to establish a conundrum for the introduction of novel, off-platform unit operations. If they will not be introduced in Phase I processes due to speed-to-market pressures, they must be delayed to the Phase III process. Yet the introduction of significant process changes (especially those that could negatively affect the impurity profile of the product) after Phase I safety studies is a high hurdle that may require additional clinical trials, increasing the cost of the drug development and potentially delaying product launch. The introduction of new technology after

licensure may appear to have an even higher bar, even if the risk of an untoward effect of process changes is very low. There have been instances of significant post-licensure changes in purification processes for recombinant proteins (10–12), which in some cases required additional clinical studies. The U.S. Food and Drug Administration (FDA) allows process changes to be managed under comparability protocols without clinical trials if the process change has no impact on product safety, potency, or efficacy, and if the product is well characterized.

A major benefit of establishing a purification process platform is realized when a reasonably long period elapses without significant changes that alter process development or manufacturing operations. This leads to an approach whereby process improvements are bundled and introduced through a controlled, internal review and decision-making process (a “punctuated equilibrium” for the platform). The evolution of a company’s platform is a healthy process, as superior separation media and accumulated development knowledge combine to offer significant advantages in development speed, reduction in COGs (see Chapter 9) or improved plant productivity. The change-control process for the platform should be managed to prevent changes from occurring too frequently, while allowing the introduction of clearly superior technology when appropriate.

## **1.4 TYPICAL UNIT OPERATIONS AND PROCESSING ALTERNATIVES**

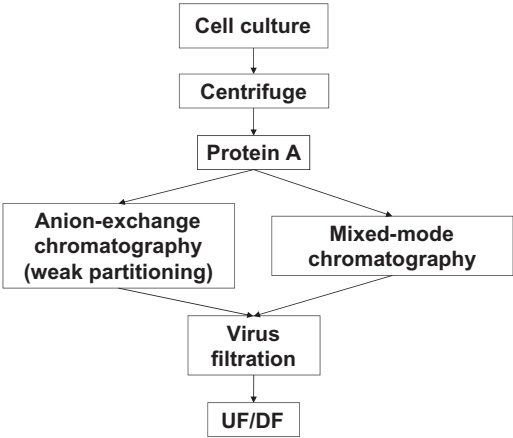
Centrifugation is currently the method of choice for harvest operations (13, 14). Continuous-flow disk-stack centrifuges are robust, are easily scaled across various fermenter volumes, and use a relatively generic set of processing parameters. It is unlikely that centrifugation can provide a sufficient degree of solids removal to allow the centrate to be processed directly by the initial capture chromatography without further filtration, although processing alternatives such as flocculation may offer a significant reduction in the filtration area required (15). Depth filters may remove protein [host cell protein (HCP) or product] during filtration (16), and changes to filtration media should be evaluated carefully. Centrifugation parameters can affect the filter area needed and can influence the colloidal properties of the feedstream. Therefore, consideration of both centrifugation and filtration parameters is required for optimal performance and integration of these steps. Although centrifugation is likely to remain the preferred choice for large-scale operations, smaller-scale cell culture harvests ( $\leq 400$  L) can also be performed using a combination of depth and size filtration. Newer technologies, such as pod harvest units, make filtration a more attractive option at small scales.

The majority of processes use Protein A chromatography as the initial capture chromatography step. Two potential issues associated with Protein A

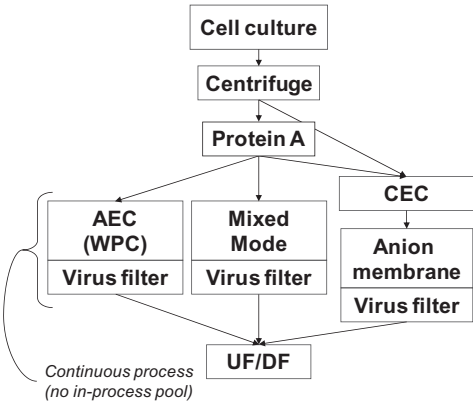


chromatography are leaching of the Protein A ligand from the resins into the product pool and the higher cost of Protein A resins as compared with nonaffinity resins. Alternative ligands to Protein A that are either small molecules or polypeptide mimetics of Protein A have generally not delivered increased load capacity while maintaining selectivity (17). Most of these resins are not known to be under active investigation in industrial laboratories. Other alternatives to Protein A chromatography as a capture step typically include ion-exchange chromatography, and because many mAbs have pIs greater than 7, CEX resins are the primary choice (see Chapter 5). With CEX, the pH and/or conductivity of the harvest cell culture fluid may need to be adjusted, and in some cases the product in the feedstream should be concentrated using ultrafiltration, to obtain optimal capacity and throughput. This conditioning operation should be evaluated when the overall costs of affinity capture are compared to those of nonaffinity capture. The lower pH and conductivity may affect antibody stability in the feedstream (because of acid-activated proteases) or precipitation of proteins (either product or HCP), thus necessitating increased filter area. However, precipitation of HCP during conditioning may contribute to HCP removal during the process (see Chapter 5), although it could cause complications during ultrafiltration. The potential of impurity precipitation as an up-front conditioning step is addressed later (see Chapter 15), although the economics of the technique do not appear to be favorable, at least at the high concentrations of the precipitant evaluated by those authors.

The number and types of polishing steps will be determined by the nature of the product and process-related impurities. In general, a Protein A pool will be more pure than a nonaffinity pool. Cell culture conditions that increase expression but also result in higher levels of aggregate or charge variants may influence the choice of affinity or nonaffinity process or may require additional polishing steps. A nonaffinity process is generally less amenable to a platform approach because not all antibodies bind well to the capture column without custom feedstream modifications, as in the case of CEX resins. Furthermore, a Protein A capture step offers greater freedom to match the subsequent processing step without feedstream adjustments (see Chapter 4). If the affinity pool is relatively pure, only one additional chromatography polishing step may be necessary. There have been platforms that use just two chromatography steps, where the AEX step is run under conditions of weak partitioning (8), as shown in Figure 1.4. Two-column processes offer advantages beyond the obvious cost reduction associated with eliminating a step from the process (see Chapter 5, which provides examples of processes comprising two columns plus a membrane adsorber). New, mixed-mode chromatography resins may also aid in minimizing the number of polishing steps by increasing the separation performance for each resin (see Chapter 6). Product-related impurities with only slight charge or size differences can be the greatest challenge for the purification process because of their similarity to the product. Controlling these impurities to acceptable levels during the cell culture process facilitates a two-column process.



**FIGURE 1.4** Emerging two-column platforms.



**FIGURE 1.5** Future high-efficiency platforms. AEC = anion-exchange chromatography; CEC = cation-exchange chromatography; WPC = weak partitioning chromatography.

Future platforms could build on continuous processing, two-column processes, and alternative unit operations (Figure 1.5). Alternatives to chromatography may simplify processes, reduce costs, and facilitate the processing of large batches. Currently, binding capacities for adsorptive membranes are lower than those of resins, making them most suitable for flow-through chromatography operations in which relatively low amounts of impurities are bound. Membrane adsorbers could replace an ion-exchange polishing step and may offer reduced buffer consumption and small pool volumes when compared to resin column chromatography (18). They may also be more conducive

to a continuous process in which the feedstream moves through each unit operation without the collection of a discrete pool, although pH and conductivity targets must coincidentally match subsequent unit operations. Other alternative technologies include crystallization and precipitation (9) (see Chapter 14). For individual antibodies, these technologies may provide a performance benefit in cost or productivity that would merit their implementation in a process. However, because of mAb variability, they are not likely to be amenable to a platform process, and they require additional resource investment during process development. Factors to consider in evaluating such novel process options include annual production requirement, expression level, COGs target, and production costs. As outlined later (see Chapter 12), economic considerations must include capital investment, operating expenses, economies of scale, the impact of opportunity costs of extended changeover periods, and the processing success rate.

The concentration of drug substance is linked to the requirements for drug product. For intravenous delivery, the drug product concentration may only be 5–20 mg/mL. Drivers for concentrations of 100 mg/mL or greater include the benefits of subcutaneous administration and a reduction in the number of drug substance storage vessels (often large stainless steel tanks) for very large batches. Achieving concentrations greater than 100 mg/mL depends on the solubility of the antibody as well as the formulation excipients, concentrations, and pH. Operational challenges to high-concentration formulations include mAb stability during concentration and diafiltration, product losses due to system hold-up volume and incomplete recovery, and the extreme viscosity of some antibodies as the concentration increases. These challenges may be addressed by careful equipment design and by a formulation that minimizes viscosity and maximizes product stability.

## 1.5 VLS PROCESSES: TON-SCALE PRODUCTION AND BEYOND

The recent increases in cell culture titers are likely to continue as cellular productivity and cell density improve and as production phases are extended. Titrers as high as 10 g/L have already been reported (19), and titers of 2–5 g/L or more will be common. The rise in titers combined with predictable scale-up to larger bioreactors has sparked debate about purification bottlenecks that limit a production plant's capacity. However, efforts to intensify the purification process should stave off capacity limits for all but the largest-volume products (20, 21). The benefits of chromatography resins and ultrafiltration membranes with elevated capacities and improved volumetric productivities allow purification processes to keep pace with advances in cell culture. The most common limitations are buffer make-up and storage capabilities, in-process pool tank volumes, and the scale of the production equipment. High-capacity resins and membranes reduce buffer consumption, generate

high-concentration in-process pools that minimize process volumes, and allow larger batches to be processed with existing equipment. A two-column process is significantly more productive than three-column or four-column purification trains, and for almost all products, it should enable VLS production without constraint.

Increased cell culture titers generated by extended fed-batch operations and high cell densities give rise to very large batch volumes (e.g., a 10 g/L titer in a 25,000-L bioreactor would generate 250 kg of harvest material and a 200-kg batch of drug substance). An extremely long bioreactor production phase affects facility utilization and staffing. At this scale, very few batches would be required to supply the market for most mAbs. A 1-ton demand would be met with only five batches, requiring very little production time in a plant operating multiple bioreactors. Efforts to level the staffing load or to manage product inventory may result in the operation of production bioreactors at less than maximum volume, if plant capacity is not appropriately matched to demand (this constraint is relaxed if the mAb is produced by a CMO). Furthermore, as the bioreactor production stage lengthens, the mismatch between the shorter cycle of the downstream process and the bioreactor becomes more problematic (22). Harvesting a single reactor for an extended culture (e.g., every 24 d or more) and using a 3-d purification process means that the purification staff may be unoccupied for a large portion of the interval between harvests. While this ratio allows a single purification train to service eight bioreactors, the enormous production capacity of a plant running high-titer processes would invariably result in inefficiencies unless the plant is also making other products.

Purification costs are the dominant drivers for VLS processes, but they are still low when expressed on a per-gram basis. Key COG components are the Protein A resin and the virus filter. Currently, the reuse of high-capacity Protein A resins for 200 cycles or more (23) reduces costs associated with this unit operation to approximately \$1 per gram. This analysis presumes that the resin is used to its full lifetime, which will be the case for large-volume products in steady production over several years. When demand does not require enough runs to extend the Protein A resin to its validated lifetime, COGs will increase, with the cost per gram declining over time as the initial investment in the resin is diluted over successive batches (e.g., if a 100-kg annual demand can be produced with two 50-kg batches, each requiring five loading cycles for the Protein A step per batch, the resin will not reach 200 cycles of use until 20 years after launch, so limits on chronological resin age almost certainly dictate a shorter life span). Another significant purification cost is the VF filter. Provided that the membrane can offer reasonable fluxes with highly concentrated feedstreams, an extended operation of several hours will minimize the necessary membrane area and the cost. The reuse of VF membranes is attractive, especially for new membranes that are developed to withstand harsh sanitization cycles and do not require destructive post-use integrity testing.

## 1.6 PROCESS VALIDATION

Process validation of purification processes defines process parameter ranges, critical process parameters, virus removal, membrane and resin lifetimes, and process pool stability. Control ranges ensure that the goals of the unit operation, including the appropriate product quality, are met. The variability of the incoming feedstream, the capability of the downstream process steps, and the potential interactions of all parameters for each step must be considered. A chromatography step may have 10–20 operating parameters. A series of designed experiments in which all parameters are examined would result in an impossibly large study. By applying an understanding of the process, along with a risk assessment, parameters can be placed into two categories: those that may have interactions with other variables and those that can be studied in single-parameter studies. For an ion-exchange column, examples of the potentially interactive variables include elution pH, elution conductivity, column loading, and temperature. Single-parameter studies might include flow rate, bed height, wash volumes, and pooling conditions. The risk assessment should contain the rationale for the type of study chosen for each parameter.

The process platform also offers significant benefits during process characterization and validation. Modular validation leverages data from previous studies to support the control ranges for a new antibody (24). The criteria that allow a modular approach should be defined, as should the parameters that qualify for modular or product-specific validation. For example, if a study for antibody X had shown that Protein A bed heights between 15 and 25 cm had no effect on the process, that study could be used to support the same bed-height range for antibody Y. However, because different antibodies have different dynamic binding capacities on Protein A resins, a product-specific study for the load range would be necessary. Unit operations that tend to be less product specific, such as centrifugation, virus filtration, low pH virus inactivation, and UF/DF, are the most amenable to the modular approach.

The quality by design (QbD) initiative is a comprehensive approach to product development and life cycle management in which the product and process parameters are designed to meet specific objectives. A thorough understanding of process parameters and their interactions and impact on product attributes defines the design space (25). Once approved, this design space offers regulatory flexibility for post-approval process changes. Validation studies must address the input variables (e.g., impurity levels) to the unit operation from the preceding step. Modular validation complements QbD and the definition of design space for platform processes. A company can use data from validation studies on similar molecules, combined with risk analysis, to define the design space for a related molecule. As a company accumulates data on several mAbs, it may be possible to develop a master file that provides the foundation for the design space for each new mAb manufacturing process.

For mAbs produced in mammalian cells, validation of the purification process to remove or to inactivate adventitious viruses is a requirement (see

Chapter 8). In many cases, the platform processes for mAb manufacturing have an exemplary viral safety profile: they use cells, such as Chinese hamster ovary cells, which do not express viable retroviruses (26); use a culture medium with no animal-derived raw materials; include viral safety tests and procedures for postproduction cells for each lot; and establish the capability of the purification process to clear or to inactivate viruses. The nature and scope of these studies—including the number and types of viruses studied, determination of the overall clearance of the model viruses, and confirmation of the validity of the scale-down model and of the ability of resins to remove virus over their claimed lifetime—are described in the International Conference on Harmonisation Q5A (27). The concept of modular viral validation was first established by the FDA in 1997 (28). It is critical to establish criteria for the use of modular validation data. As an example, for a chromatography step, these criteria should include the exact resin type, its position in the process, the robustness of performance, and the comparability of process parameters (e.g., flow rate, protein load, and bed height).

An understanding of the mechanism of virus removal or inactivation is an important element of the modular approach. The design space for a step is established by assessing the effect of process parameter ranges on product purity (e.g., product variants, aggregates, and HCP). If a step contributes to viral safety, an understanding of how these parameter ranges affect virus removal is also needed. However, virus removal validation studies are complicated and expensive. Therefore, studies are performed at the set point for process parameters. To address this issue, modular data from a comprehensive study on the effect of process parameters on viral clearance performed for one mAb (to define its design space) are used to support the design space for subsequent mAbs. With the mechanism of action and process robustness established, companies may make modular claims for pH inactivation and VF (29). Typically, the other step that contributes to viral safety is AEX chromatography. If this step is amenable to modular claims, then there may be no need to conduct product-specific viral clearance studies for clinical studies if the extent of viral clearance from all modular studies is great enough to provide a sufficient safety factor.

Even when modular validation can be applied, unique studies will be required for each mAb. The hold times for conditioned media and in-process pools will be a unique combination of the feedstream, impurities, and the mAb sequence and structure. Forced decomposition studies should be performed to identify degradation pathways. Resin reuse studies are unlikely to be amenable to a modular validation approach because resin lifetime will be dictated by unpredictable effects of the antibody's properties as well as the impact of feedstream variability arising from the unique characteristics of individual cell lines.

## 1.7 PRODUCT LIFE CYCLE MANAGEMENT

Whereas the preapproval process development phase typically lasts 5–7 years, the post-approval commercial manufacturing life cycle may extend to 20 years or more. The manufacturing life cycle includes post-approval process changes, transfer to new manufacturing sites, and possibly process scale-up. The breadth and depth of process development, characterization, and validation can greatly facilitate these changes. The reasons for making post-licensure changes are varied, but the most common motivation is to increase production capacity to meet increased demand. The approaches to increased capacity take a variety of forms and present different technical and regulatory challenges. Transferring the process to a new manufacturing facility at a similar scale is the most straightforward approach, but if the second facility is not an exact fit for the process, adjustments may be necessary. Changes in scale are usually associated with a facility change. If the scale-up of the cell culture and purification processes is not aligned, particularly if the purification equipment is undersized relative to cell culture, then it is likely that process adjustments will be necessary.

The interval between the definition of the cell culture and purification processes and product approval may be several years. During this time, advances in cell culture technology may enable increases in titers of 50% or more, which could be used to increase productivity of current products through post-licensure process changes. The effects of this increased titer on the purification process and facility are far-reaching and will probably require both process and equipment changes. If no changes are made to the operating conditions of the purification process, direct scale-up requires increases in column diameter and volume, membrane and filter areas, flow rates, and buffer and pool volumes. For facilities designed several years prior to the process improvement, the maximum titer used as a basis for the plant design is typically lower than that provided by the newest product.

Before process changes are made, a thorough assessment of the locations of purification bottlenecks is necessary. Changes to equipment, although perhaps more straightforward from the technical and regulatory perspectives, may be constrained by space availability, plant downtime during retrofitting, and capital expense. Purification process changes fall into two general categories. The first category includes modifications to optimize wash and elution volumes, to narrow pooling conditions, and to increase column capacities. How the ranges for these parameters are specified in the license will determine the regulatory pathway to the implementation of these changes. The second category of changes involves substantial modification to the process and requires regulatory approval prior to implementation. High-capacity chromatography resins can be used to address process bottlenecks. Pool-volume reduction affects both the step's pool volume and the downstream pool volumes if that step is operated in the flow-through mode. Because one main facility limitation is tank number and volume, changing the order of unit operations to minimize



the effect of pH and conductivity adjustments for downstream steps can be beneficial. Of course, adding UF/DF steps is a one way to address tank limitations, but this change involves new capital equipment, downtime, and space considerations.

Increases in the product titer may not be the only changes to the feedstock. Modifications to the cell culture process may also change the product and process impurity profiles. Other changes to the feedstock include increased debris load with different physical properties, which will challenge the harvest operation. Evolving regulatory requirements are also drivers for process changes. These regulatory drivers may not be limited just to requirements of the national and international regulatory agencies [e.g., the FDA and European Medicines Evaluation Agency (EMA)]; they may also include state and local agencies that govern areas such as hazardous waste and discharge into the local water system.

Risk assessment should assess the scope of process characterization work needed to support process changes. This assessment should evaluate the impact of the changes on the overall process and the applicability of validation studies from the original process. For example, if changes were made only to the cell culture process, the Protein A step would need to be revalidated. If the Protein A pool from the new process was comparable (i.e., had the same level of product and process impurities), then the unit operations downstream of Protein A would not need to be revalidated.

Commercial manufacturing is critical to the patients who rely on these drugs, and uninterrupted product supply is dependent on the availability of raw materials. For many purification raw materials, such as chemicals, supply is not critical because multiple suppliers can meet the necessary quality and quantity requirements. However, for some raw materials (e.g., chromatography resins), similar raw materials are not interchangeable among vendors. During process development, a specific resin is chosen for each step and the process optimized for that resin. Changing to a similar resin may not result in the same purity and yield under the same operating parameters. Certain membranes and filters may require unique operating conditions. For these process-specific raw materials, it is critical to have a risk-mitigation plan that will ensure uninterrupted manufacturing should a vendor be unable to supply a raw material (30). The risk can be mitigated by carrying an additional inventory of these raw materials or by developing processes with alternative raw materials. When alternative raw materials are used, the product quality of the drug substance must still meet the appropriate specifications. Ideally, an alternative raw material will provide the same process performance as the original raw material, although other factors (e.g., cost, ease of use, and process robustness) may make it a second choice. If the alternative raw material does not result in the same product quality in the intermediate process pool, it will be necessary to assess whether downstream steps can achieve the same product quality either with or without modification. Once alternative raw materials have been identified, an implementation strategy should be developed. The



cost of the work necessary to develop, characterize, and validate an alternative raw material should be balanced against the cost of carrying an additional raw material inventory. Other factors to consider include the resin's lifetime, cost, and annual demand. When a raw material is used in more than one process, cumulative use and business risks also need to be considered. A platform process that relies on a small number of raw materials minimizes the number of raw materials in inventory and may reduce the overall backup required. However, this approach increases business risk, because a supply disruption may affect many products.

Raw materials may be grouped into classes such as chromatography resins, sterilizing filters, virus filters, and ultrafiltration membranes. The degree to which such raw materials can be substituted in these classes can be considered according to their intended use. For example, there may be significant differences among CEX resins in terms of resolution, with each resin requiring distinct operating conditions. These differences are based on the unique physical (e.g., bead and pore size) and chemical (e.g., ligand and resin matrix) properties of resins. Alternatively, sterilizing filters have a clear mechanism of separation (a size differential), and the operating parameters or design space can be defined to cover various filters. Taking this a step further, as the QbD concept evolves and design space is increasingly defined on the output of a unit operation, it may be possible to use different raw materials for a step as long as the output of the processing step (e.g., product purity and isoform distribution) is maintained.

For post-approval process changes, QbD and design space can greatly facilitate such changes. Knowledge gained during process development leads to a thorough understanding of process input ranges and process outputs, particularly for critical quality attributes, and thus lays the foundation for a robust design space. Such a design space is the basis for consistent, reproducible manufacturing operations, but also allows changes within the design space over the product's life cycle in response to changing conditions.

## 1.8 FUTURE OPPORTUNITIES

With standard mAb purification platform processes firmly entrenched in the industry and representing the current state of the art, one might ask what the future will hold. Several opportunities addressing this question will be presented in this section. Some of these suppositions may come to pass, while others may never be adopted.

Because mAbs are a group of compounds large enough to warrant consideration as a separate class (28), the adoption by regulatory agencies of common targets for impurity levels could be of great value to the industry. These targets would define process development design principles that are generally regarded as safe and would represent impurity levels that are readily achievable with standard processes. Currently, the only impurity that has a well-accepted limit

is host cell DNA. The FDA initially issued guidance that specified 10 pg/dose (31), and then 100 pg/dose (28), and this was followed by World Health Organization guidelines that specified 10 ng/dose (32).

At the 2003 Well Characterized Biologicals conference, FDA representatives cited typical levels of HCP, aggregates, and residual Protein A levels for mAbs described in Phase I Investigational New Drug Applications (33). Although levels of process- and product-related impurities have not been translated into regulatory guidance documents, the presentation suggests these levels are generally regarded as safe with the caveat that dose, dosing frequency, and route of administration are all important factors have not been issued in regulatory guidance documents, and while they do not reflect a common international position, the presentation of these levels hints at the possibility of establishing limits that are generally regarded as safe for these host cell and process-related impurities. Clarity regarding these targets could greatly simplify process development, enable more productive processes that do not “overdevelop or overengineer” without compromising product safety, and aid in the regulatory review of both clinical filings and commercial license applications.

Of course, the appropriate limits on impurity levels should be evaluated on a case-by-case basis, with risk adjustment for therapeutic dose, disease indication, dosing frequency, and other clinical factors. Ideally, a set of common target impurity levels would cover the most conservative case, so that adjustments would generally be made only to increase the impurity levels if warranted by process capability, following a thorough review of the clinical context for the product. In the opposite case, companies would be expected to recognize the highly unusual combination of factors that would warrant a reduction in the target impurity level in order to lower the risk of adverse clinical events.

Programs are under development at several companies for identifying, early in the discovery phase, mAbs that have inherent processing or quality issues, such that mAbs directed at the same target but with alternate sequences (and thus improved characteristics) could be chosen instead. Examples of the product attributes that may be included in a manufacturing feasibility assessment include stability (both drug substance and drug product), maximum solubility and suitability for subcutaneous administration, viscosity at high concentrations, tendency toward unwanted microheterogeneity, and compatibility with a platform purification process. Recognizing that the value of many of these attributes does not manifest itself until late in a product's life cycle, early intervention based on the feasibility assessment prior to the initiation of clinical trials is a prudent approach to accommodate manufacturing objectives.

Several companies are evaluating production hosts other than mammalian cells, including transgenic animals or plants, recombinant yeast engineered to express the appropriate glycosyltransferases and thus provide natural oligosaccharides, and full-length mAbs from *Escherichia coli*. It is unlikely that the purification platform developed for mAbs expressed in mammalian cells will transfer cleanly to mAbs produced by these alternate hosts. The up-front

clarification and capture steps may be most affected by the change in the nature of the feedstream, yet the polishing and final UF/DF steps will probably be largely unaffected. The assurance of viral safety will not be an issue for mAbs derived from microbial sources, yet may be a concern for those from transgenic hosts (even plants, if they are grown in an uncontrolled environment).

The dynamic binding capacity for Protein A resins is currently 30–50 g/L, which offers significant advantages for the processing of high-titer feedstreams when compared to older Protein A media that had capacities at or below 20 g/L. Increasing the dynamic binding capacity to 80–100 g/L, to levels commensurate with ion-exchange resins, would result in even smaller in-process pools, reduced buffer consumption, and potentially lower COGs. Although the practical limit to Protein A resin capacity may fall short of 100 g/L, product capture using ion-exchange resins could offer even higher capacities, albeit probably at the expense of general applicability and streamlined development of Protein A chromatography for product capture.

There would be a great advantage in the development of high-flux, high-capacity parvovirus filters, which would significantly decrease COGs and increase productivity. Reusable virus filters could also significantly affect COGs. For a process with a nonfouling feedstream and a control strategy using consistent process flux decay and clean water flux restoration as important process parameters, combined with a validated post-use integrity test, companies may consider developing validation packages that support virus filter reuse. Technology to inactivate viruses (heat or ultraviolet irradiation) that could potentially substitute for VF or pH inactivation would entail capital costs for new equipment, and could result in scale-up issues.

A major benefit to COGs and manufacturing flexibility could be gained by sharing Protein A resins and membranes between products. Commercial facilities would be able to use resins and membranes to their full lifetime and to enjoy reduced plant changeover losses. For clinical production, the impact on development costs would be even greater—tens of millions of dollars are spent each year on clinical Protein A resins that are used just a few times and then sent to a warehouse, many never to be used again. Accurate and sensitive methods to evaluate product-specific carryover are necessary to ensure that the risk of cross contamination of products is minimized. The testing methods would be combined with a concurrent validation strategy providing direct evidence that the resin had minimal product carryover, perhaps to the standard used for the processing of small molecules using shared compounding equipment (one thousandth of a dose carryover limit). This approach would likely be established on a company-by-company basis, since the testing methods and ability to clean the resin between products would be functions of the unique combination of product characteristics, resin type, and regeneration scheme.

The extent to which Process Analytical Technology (PAT) will be applied to mAb purification is a controversial topic (34). It seems unlikely that an inherently robust platform process, as demonstrated by process

characterization for several different mAbs, would require many unit operations using PAT. One potential example of PAT is control of the Protein A column load capacity through the direct measurement of product losses during the load cycle, allowing maximum column loading with each cycle and accommodating changes in dynamic binding capacity with increased column lifetime (23). Alternatively, control could be managed by periodic off-line measurements that would risk little product yield given a slow decline in column capacity. Another solution would be to set the column's load capacity at a more conservative level, which might require only an additional Protein A cycle to process a batch and would not need any measurements or control strategies whatsoever.

## 1.9 CONCLUSIONS

In the more than 20 years since the licensure of the first mAb, antibody purification technology and processes have matured significantly. Platform processes have codified both process designs and development activities at many companies and will have an even more significant impact on the industrialization of commercial mAb production as more mAbs are produced in multi-product facilities. Advances in process understanding have combined with the introduction of high-performance separation media to enable the rapid definition of increasingly productive processes that yield very pure drug substances.

Through the cumulative impact of high-titer cell culture processes, streamlined purification processes preventing plant productivity bottlenecks, and flexible operations enabling ready transfer of processes between production plants, mAbs will emerge as a special class of therapeutics and will become the least expensive biologics to manufacture, exploiting economies of scale, manufacturing flexibility (both within and between facilities), and common supply-chain elements. This shift may open up the potential for new clinical indications for mAbs, e.g., for the treatment of disease indications that may require high doses and long-term administration, and may affect large patient populations. The drug substance supply for a blend of both blockbuster as well as smaller, niche products could be accommodated by flexible sourcing options afforded by both internal and contract manufacturing, provided that the processes used are based on a common design that is readily transferable to plants configured to run all subtle variations of a standard mAb purification process.

In one possible future, there will be an excess of cGMP mAb production capacity in the global supply network. All plants will use homologous purification processes based on current platforms that have been updated with modern separation media and refined process designs. This supply network would mirror a decades-long period in the history of global IgIV production, during which there were multiple plasma processing plants worldwide, all of which

ran slight variations of the Cohn process. Although each plant had slightly different designs, production capacities, and product mixes, the plants were generally more similar than different. The maturation of recombinant mAb processing (both upstream and downstream), combined with the substantial advances in separation technology from the 1950s to the twenty-first century, makes this future state a distinct possibility.

## 1.10 ACKNOWLEDGMENTS

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# 2

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## THE DEVELOPMENT OF ANTIBODY PURIFICATION TECHNOLOGIES

JOHN CURLING

### 2.1 INTRODUCTION

The end of the nineteenth century was an era of intense activity in the history of antibody development, with the discovery of antitoxins (immunoglobulins) to tetanus and diphtheria by Emil von Behring, and Paul Ehrlich's studies of the toxin–antitoxin reaction and subsequent product concentration and standardization. This probably marked the introduction of characterization into biopharmaceutical product development. The first therapeutic serum was marketed in 1894 by Hoechst, with whom von Behring worked closely. The antitoxins were the first biopharmaceutical products, and were introduced in the USA and elsewhere in 1913. Ehrlich's work was also significant in the history of antibody purification since he investigated the nature of the antigen–antibody reaction and the role of complement and recognized that dyestuffs had specific affinity for certain cellular components.

Although precipitation was described in the 1850s, the relationship between protein solubility and neutral salt concentration was first investigated by Hofmeister in 1888 (1). In 1893, Brieger and Ehrlich (2) described the use of ammonium sulfate to precipitate an immunoglobulin from the milk of a goat immunized against tetanus, and thus some of the techniques employed in monoclonal and polyclonal antibody purification today have a history that goes back over 100 years. Most antibodies precipitate at ammonium sulfate concentrations above 50% saturation. The fractionation or “salting out” of immunoglobulins with the neutral salts ammonium sulfate and sodium sulfate



combined with the acridine dye Rivanol<sup>®</sup> was discussed by Heide and Schwick in a 1973 review (3).

The first approved monoclonal antibody (mAb), Muromonab-CD3 (Orthoclone OKT<sup>®</sup>3), is processed from murine ascites by ammonium sulfate fractionation followed by ion-exchange (IEX) chromatography (4). The use of ammonium sulfate precipitation for mAb purification was evaluated as an initial step and was compared to gel filtration, hydroxyapatite chromatography, anion-exchange (AEX) chromatography, and affinity chromatography on immobilized Protein A by Manil and colleagues in 1986 (5) who, not surprisingly, found Protein A capture to be the most efficient protocol.

Sodium sulfate precipitation of an ovine polyclonal antibody was used as the initial step in the original purification scheme for the full-length intermediate of the Fab fragment crotalid antivenom CroFab<sup>™</sup>, but was replaced by a direct capture step employing the synthetic ligand adsorbent MABsorbent A2P<sup>®</sup> (6). The current process uses mixed-mode chromatography (hydrophobic plus IEX; see Chapter 7) as a capture step (7). A similar polyclonal product, an ovine immune digoxin Fab fragment (DigiFab<sup>™</sup>), is manufactured using a combination of precipitation and affinity chromatography (8).

Precipitation can be used to direct either the target antibody or the protein impurities and potential contaminants to the precipitate or the supernatant, the precipitate generally being preferred for the antibody since this step simultaneously removes water and concentrates the product (see Chapter 9). In the isolation of a specific protein from a complex source, such as blood plasma, it is common to use differential precipitation in a sequence of conditions. The use of ethanol to precipitate proteins at their isoelectric points in the large-scale production of blood plasma products was developed by Cohn and colleagues (9). The original method 6 targets albumin, which was needed in the greatest quantities in the 1940s, whereas a method 9 was developed by Oncley (10) for the isolation of the immunoglobulin fraction. The immunoglobulin is precipitated in a combined fraction II + III using 25% ethanol at  $-5^{\circ}\text{C}$ , 3% total protein concentration, pH 6.9, and 0.09M salt. In a Kistler–Nitschmann modification of the method, the immunoglobulin is precipitated using 19% ethanol (11, 12). Yields should be in the region of 70% with >50% purity. However, recoveries may be poor due to partitioning between the solid and liquid phases and/or inefficient liquid–solid separation (13). Ethanol has antibacterial properties but is also a denaturant, so it is not used in contemporary mAb purification processes.

Early studies of precipitating agents included the investigation of short-chain fatty acids, particularly caprylic acid (*n*-octanoic acid) (14). Steinbuch (15, 16) developed a method to isolate IgG from human plasma so that the IgG remained in a solution at pH 4.8, using 68g caprylic acid/L of plasma. Further purification was accomplished either by IEX or by ethanol precipitation. Caprylic acid is only useful for processes in which the target protein remains in the supernatant because protein–fatty acid complexes formed in the precipitate are difficult to dissociate. After many years without an indus-

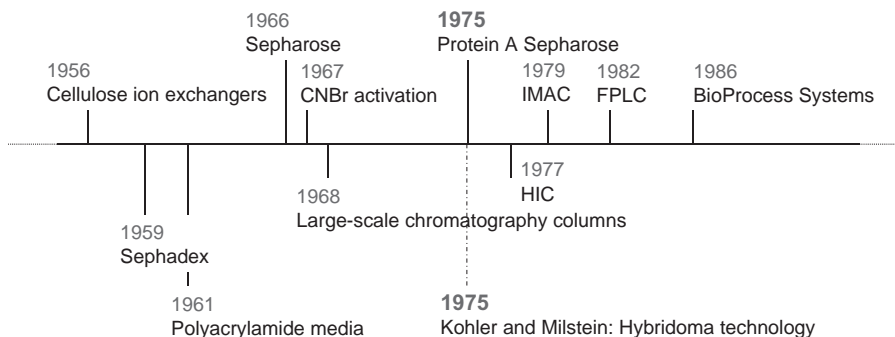
trial application, possibly because caprylic acid is a poor target protein precipitant, it is now used to remove non-IgG proteins in the industrial-scale process for the intravenous immunoglobulin (IVIG) product Gamunex™ (17). Similarly, the method described by Parkkinen (18) is now used commercially. It is also well established that caprylate can efficiently inactivate lipid-enveloped viruses (19) adding to the reasons for its preferential use over polyethylene glycol.

Caprylic acid precipitation of nonantibody proteins in ascites was first described by Russo (20). Various methods were developed based on caprylic acid, caprylic acid in combination with ammonium sulfate, and precipitation with IEX and high-performance liquid chromatography (HPLC) (21–23). The authors noted problems associated with caprylic acid such as reduced affinity for certain antibodies, loss of monomeric IgG, high residual albumin levels, and low recovery of antibody activity, suggesting limited applicability in the industrial processing of mAbs.

In many areas of protein and plasmid DNA purification, different research groups have sought to develop nonchromatographic methods to achieve simplicity, scalability, economy, and independence from resin manufacturers. Contrary to Mohanty and Elazhary (23), Reik and colleagues (24) described a process suitable for large-scale production that used caprylic acid precipitation of impurities followed by ammonium sulfate precipitation of the IgG, thus significantly reducing the albumin content. Yields over the precipitation step of 50%–80% explain why this process is not used despite its ability to prepare IgG1, 2a, and 2b class antibodies.

## **2.2 CHROMATOGRAPHIC PURIFICATION OF ANTIBODIES BEFORE PROTEIN A**

Adsorption and displacement chromatography, and later use of hydroxyapatite, were first described by Tiselius (25). He summed up the importance of partition chromatography in his presentation speech for the Nobel Prize in Chemistry (1952), awarded to Martin and Synge (26): “This tool has enabled research workers in chemistry, biology and medicine to tackle and solve problems which earlier were considered almost hopelessly complicated.” The work of these mid-century laureates followed the investigations of Mikhail Tswett, who described the principles of his separation techniques as applied to plant pigments in 1903, but first used the term “chromatography” in 1906 (27, 28). Even so, it was not until the introduction of hydrophilic matrices 50 years later that chromatography really found a place in protein purification. The introduction of cellulose ion exchangers by Peterson and Sober (29) in 1956, cross-linked dextrans (Sephadex®) by Porath and Flodin (30) in 1959, and polyacrylamide in 1961 and agarose in 1964 by Hjertén (31, 32) initiated a revolution in protein chromatography. However, the adoption of cellulose-based resins was restricted, largely due to variations in the physical and



**FIGURE 2.1** Introduction of chromatographic media during the period 1956–1986. The time line shows the techniques available when the first monoclonal antibodies were in development. Protein A Sepharose was introduced in 1975, the same year in which Kohler and Milstein published their work on hybridoma cell culture (130). Historical data were provided by GE Healthcare Bio-Sciences, Uppsala, Sweden.

chromatographic properties of diethylaminoethyl (DEAE) celluloses. The time line for the commercial introduction and availability of products developed principally at Pharmacia Fine Chemicals (now GE Healthcare Bio-Sciences) and typically used agarose as the support matrix (Fig. 2.1).

Initially, serum fractionation was used to demonstrate the efficacy of chromatography, first through slow and laborious procedures using Sephadex G-200 (33), and then by AEX chromatography on DEAE-Sephadex (34). A batch technique to overcome the shrinking/swelling properties associated with Sephadex ion exchangers was developed (35). One of the most influential methods was published by Hoppe and colleagues in 1967 (36) followed with a 1973 modification (37), on the production of an “anti-D immunoglobulin for immunization prophylaxis of Rh negative women.” This was the first time clinical use (*intravenous* injection) of a chromatographically purified, immunoelectrophoretically pure, immunoglobulin product was discussed. The method combined initial purification on DEAE-Sephadex followed by ethanol precipitation to reduce the risk of hepatitis B virus transmission. With pilot-scale work commencing in 1976, Hoppe’s method was one of the starting points for work at the Winnipeg Rh Institute (now Cangene Corporation) that developed WinRho® and many other hyperimmune antibody products. The procedures were entirely chromatographic and were also applied to whole, normal plasma fractionation for IgG and albumin (38, 39). In 1969, a new type of quaternary anion exchanger (QAE) was introduced, produced by the hydroxypropylation of DEAE-Sephadex, which overcame some of the issues associated with swelling and shrinkage. A monomeric, immunochemically pure immunoglobulin was produced by a two-step process using Sephadex G-25 and QAE-Sephadex (40). These early, IEX-based methods were the platform upon which the application of process-scale chromatography in mAb produc-

tion was built. An industrial batch method using DEAE-Sephadex as a polishing step in IgG production was introduced by Björling in 1972 (41). At the Fraction II stage, the immunoglobulin product is 85%–90% pure with albumin, transferring, and hemoglobin–haptoglobin complexes as protein impurities that are removed by adsorption to the ion exchanger. Additionally, the chromatography served as a virus reduction step with respect to the hepatitis B surface antigen, then known as the Australia or Au-antigen.

Cross-linked dextran products (Sephadex) were far from ideal for process use, and with the commercial introduction of Sepharose® in 1967, followed by cross-linked (CL) agarose in 1975 and Fast Flow in 1985, a new era in process chromatography emerged. These new base matrices extended the use of protein chromatography from simple desalting applications on rigid gels, e.g., Sephadex G-25, to separations (fractionation) of closely related species predominantly by IEX prior to 1975.

Collaboration between Pharmacia Fine Chemicals and the Finnish Red Cross extended the chromatographic purification of albumin (42) to the immunoglobulin fraction of plasma. The method used the Cohn Fraction II + III as a starting material, the same DEAE step as in the albumin process and introduced a serial column of lysine-Sepharose to reduce plasminogen and plasmin. The antibody fraction was then captured on SP Sepharose and was eluted at a concentration of 100–120 g/L (43). The procedure forms the basis of later methods now used in the commercial production of intravenous IgG products such as IntraGam®, in processes downstream of ethanol fractionation (44). After initial capture, flow-through unit operations are generally preferable unless the antibody needs to be bound to allow the removal of processing reagents such as solvent/detergent, as occurs in the industrial process for the normal IVIG, Gammagard® (45). Purification methods for intravenous IgG products have been reviewed by Rousell and McCue (46) and more recently by Buchacher and Iberer (47). IEX chromatography represents the main purification steps in the production of a polyvalent, polyclonal equine Fab fragment botulinum antitoxin (48).

The chromatography of proteins on tricalcium phosphate was first described by Tiselius (49), who used the expression “salting-out adsorption” in 1951. Stepwise protein elution using increasing salt concentration at constant pH on a modified adsorbent {hydroxyapatite,  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]}$ , was described a few years later (50), and Hjertén described the purification of immunoglobulin and other proteins as well as subfractions from normal serum on the same adsorbent in 1956. High buffer concentrations (>0.3 M potassium phosphate) were needed to elute some proteins, and displacement effects were observed. Thirty years later, Stanker and colleagues (51) described a single-step procedure to capture mAbs from ascites with only low residual levels of murine albumin, transferring, and other ascites proteins. In a comparative study with HPLC on Mono Q® and hydrophobic interaction chromatography (HIC) on TSK Phenyl 5PW, it was found that Bio-Gel® Hydroxyapatite (HPHT) provided fewer peaks (lower resolution) than the other chromatographic

modalities (52). This was an analytical purification investigation of an antibody to Factor VIII, which indicated a need to include ammonium sulfate precipitation in the preferred protocol.

From a biocompatibility standpoint, hydroxyapatite should be an excellent matrix for the chromatography of injectable products since it is the major component of bone and tooth tissue and is used in bone graft applications. Despite significant improvements in the matrix, particularly the introduction of beaded, ceramic hydroxyapatite, the technology is currently used in only one downstream process for an approved mAb product. This may reflect poor understanding of the mixed-mode nature of the separation, which is based on cation exchange (CEX) and calcium metal affinity, as well as anecdotal reports of difficulties with column packing. Nevertheless, Gagnon and colleagues (53) have recently addressed practical issues, integrated processes (54), and two platforms that use hydroxyapatite in either the bind-and-elute or the flow-through mode (55). Significantly, hydroxyapatite is used in these recent protocols in combination with other established chromatographic purification technologies (see Chapter 7). With the renewed interest in mixed-mode chromatography, it is possible that this technique will be used as a polishing step in future commercial antibody processes. However, it will compete with media that are specifically designed in terms of both ligand and matrix morphology to reduce host cell protein (HCP), DNA, aggregates, and antibody variants.

The introduction of affinity chromatography is usually attributed to Cuatrecasas and colleagues (56) who, in 1968 [a year after the introduction of cyanogen bromide activation by Axén (57)], described the purification of certain enzymes on “inhibitor gels” synthesized by coupling the inhibitor to CNBr-activated agarose. These authors thus introduced the notion of protein purification based on biologically functional pairs or the molecular recognition between a target protein and an immobilized partner. In 1958, Jensen reported that human serum normally contained an antibody to a cell wall protein of *Staphylococcus aureus*, which he called Antigen A (58). Grov and colleagues (59) introduced the term Protein A to distinguish it from Polysaccharide A, identified much earlier in type A (pathogenic) staphylococci by Julianelle and Wiegand (60). Interactions between Protein A and immunoglobulins were under investigation by Sjöqvist’s group, initially in Umeå and then at Uppsala University (61). This can be seen as a continuation of work at Waldenström’s laboratory, which led to the identification of Waldenström’s macroglobulinemia, a rare form of non-Hodgkins lymphoma that can now be treated with rituximab (Rituxan®) and other drugs. Chromatographic immunoglobulin purification using Protein A adsorbents is generally ascribed to the Uppsala and Lund groups led by Hjelm et al. (62) and Kronvall (63). However, a Lund University group led by Björk became known for pursuing research into Protein G derived from type G streptococci (64, 65). To quote Gagnon’s excellent text on antibody purification, “the rest they say is history” (66) or the beginning of the history of affinity chromatography on immobilized Protein A and the development of commercially viable affinity-based processes for mAbs.

Pharmacia Fine Chemicals launched its first Protein A Sepharose in 1975 and embarked on a never-ending development program as new recombinant, genetic, and molecular engineering technologies became available.

### 2.3 ANTIBODY PURIFICATION AFTER 1975

Physicochemical studies of Protein A were first published in 1972 at the same time that the use of this protein as an IgG adsorbent was proposed (67). Protein A was reported to have a molecular weight of about 42,000 Da, and later Sjö Dahl described two functionally distinct regions of the molecule. The N-terminal 27,000-Da moiety has four contiguous and very similar IgG fragment crystallizable (Fc)-binding domains (denoted D, A, B, C in order from the N-terminal), each with a molecular weight of about 7000 Da. In contrast, the 15,000-Da C-terminal region does not bind IgG but accounts for the association of Protein A with the bacterial cell wall (68, 69). The 5' sequence of the staphylococcal Protein A gene was described in 1983 and the gene cloned in *Escherichia coli* (70). The authors also identified a fifth region (E) homologous to the previously identified D, A, B, and C regions. The complete sequence, including the signal peptide and the C-terminal cell wall binding domain [region X (71)], was described by Uhlén and colleagues in 1984 (72). Later, a genetic approach was used to characterize the IgG-binding properties of Protein A and a pentavalent binding mechanism proposed (73). Elucidation of the Protein A gene sequence enabled the production of Protein A variants with different numbers of IgG-binding domains and the development of an engineered domain Z, derived from domain B but with the sensitive asparagine-glycine dipeptide replaced with asparagine-alanine (74). The alkaline stability of mutated variants of the Z domain and the development of alkaline tolerance, critical for the industrial application of Protein A, was described by Linholt and colleagues (75) and by Hober et al. (76). The genesis of Protein A has been summarized by Hober and colleagues in a recent review of Protein A chromatography (77) (see Chapter 4).

The 30 years of Protein A structure–function studies underpin the evolution of adsorbents for the affinity purification of mAbs and the close relationship between the Royal Institute of Technology in Stockholm and Pharmacia/GE Healthcare. This led to the development of different recombinant Protein A analogues, expressed in *E. coli*, which differ significantly from the endogenous *S. aureus* protein originally produced by Pharmacia/Kabi, Fermentech Ltd., and Repligen Corp. However, *S. aureus*-derived Protein A is still available from some manufacturers. Changes in regulatory guidelines and the move to eliminate animal-derived products from biotherapeutics, components used in Protein A manufacturing and any component that comes into contact with the parenteral drug product, have led to “vegan” recombinant Protein A ligands. Over the years, adsorbent matrices have become more rigid, allowing higher flow rates and shorter residence times while handling has become easier, and



high-capacity variants have been introduced from 2003 onward. Some engineered Protein A variants are coupled through the C-terminal cysteine via a thiol–ether bond to the matrix, therefore providing preferential ligand orientation and very high binding capacity, whereas a multipoint attachment analogue was developed to minimize leakage. Also by engineering five B domain repeats, binding to the Fc portion of the IgG molecule was optimized while eliminating variable region interactions (78). Protein A adsorbents that retain their variable region interactions demonstrate the need for adaptive process development (79). It is therefore important to remember that although adsorbents may bear the same generic name “Protein A,” the protein ligands differ significantly in origin, structure, and immobilization. Repligen Corp., which cloned Protein A in 1982, is a supplier of certain recombinant Protein A ligands to both GE Healthcare and Applied Biosystems (80). The main products available in 2007 for commercial processes are summarized in Table 2.1. It should also be noted that industrial processes for antibody purification have been in development since the early 1980s and that regulatory hurdles to process modifications and the well-established safety profiles of existing antibody products mean that old processes may still be based on the purification products available at the time of the biopharmaceutical development. A discussion of the current performance, selection, and use of Protein A adsorbents is discussed in Chapter 4, with performance comparisons also available in the literature (81).

Protein A adsorbents remain the capture step of choice because of the high purity (95%–>99%) and high recovery (>95%) associated with the step. Jagschies (82) reports “best-in-class” industry performance of 40–50 g/L binding capacity on Protein A resins, 300 cycles, sodium hydroxide sanitization protocols, and 70%–80% overall process yield with a titer assumption of 5 g/L culture supernatant in 2007. The contribution of ongoing adsorbent development to process intensification and a reduction in the contribution to the cost of goods in mAb manufacturing is shown in Fig. 2.2, although the data relate to single antibodies in continuous production rather than to multiple, campaign-manufactured products. In addition, buffers can be produced in-line, intermediate products transferred from one step to the next, and intermediate conditioning steps deleted. These changes and the move from three chromatographic steps to two contribute to a lowering of total cost of antibody production to the \$100–200 range. Capital investments, operating costs, and process economics have been reviewed by Farid (83) (see Chapter 20).

## **2.4 ADDITIONAL TECHNOLOGIES FOR ANTIBODY PURIFICATION**

Although affinity chromatography on Protein A adsorbents is the classic capture step, the purification train needs to ensure the reduction of HCP, DNA, and possible adventitious viruses that could influence expression of the

**TABLE 2.1 Selected Protein A Adsorbents for Fixed-Bed Chromatography, Type of Protein A, and Support Matrices**

Adsorbent	Ligand/Source	Matrix	Manufacturer	Introduced
Protein A Sepharose CL-4B	nProtein A/ <i>S. aureus</i>	4% cross-linked (CL) agarose, 90µm	GE Healthcare	1975
nProtein A Sepharose 4 Fast Flow	nProtein A <sup>a</sup> / <i>S. aureus</i>	4% highly cross-linked agarose (FF), 90µm	GE Healthcare	1982
rProtein A Sepharose Fast Flow	rProtein A <sup>b</sup> / <i>E. coli</i>	4% highly cross-linked agarose (FF), 90µm	GE Healthcare	1994
rmp Protein A Sepharose Fast Flow	rProtein A <sup>c</sup> / <i>E. coli</i>	4% highly cross-linked agarose (FF), 90µm	GE Healthcare	1996
MabSelect™	rProtein A <sup>b</sup> / <i>E. coli</i>	High-flow agarose, 85µm	GE Healthcare	2001
MabSelect Xtra™ (High Capacity)	rProtein A <sup>b</sup> / <i>E. coli</i>	High-flow agarose, 75µm	GE Healthcare	2005
MabSelect SuRe™ (Superior Resistance)	rProtein A <sup>d</sup> / <i>E. coli</i>	High-flow agarose, 85µm	GE Healthcare	2005
ProSep® A High Capacity	nProtein A/ <i>S. aureus</i>	1000-Ångström controlled pore glass (CPG), 100µm	Millipore	1990
ProSep-vA High Capacity <sup>e</sup>	nProtein A/ <i>S. aureus</i>	1000-Ångström CPG, 100µm	Millipore	2003
ProSep-vA Ultra	nProtein A/ <i>S. aureus</i>	700-Ångström CPG <sup>f</sup> , 100µm	Millipore	2004
POROS® A <sup>g</sup>	rProtein A/ <i>E. coli</i>	Polystyrene-divinylbenzene, 20µm and later 50µm	Applied Biosystems	1991, 1993
POROS Mabcapture™ A	rProtein A/ <i>E. coli</i>	Polystyrene-divinylbenzene, 45µm	Applied Biosystems	2007

*Note:* Sepharose-based products were developed by Pharmacia Fine Chemicals AB, Uppsala, Sweden, which is now part of GE Healthcare; CPG-based products were developed by BioProcessing Ltd., Consett, UK, now part of Millipore Corp., and POROS-based products were developed by PerSeptive BioSystems, Inc., Framingham, MA, USA, now part of the Applied Biosystems Group. Prefixes: n = new; r = recombinant; rmp = recombinant multipoint attachment; v = vegan.

<sup>a</sup>Original product replaced by a native Protein A (molecular weight (MW) 46.6kDa) manufactured without using any animal-derived components.

<sup>b</sup>Protein A (domains E, D, A, B, C) engineered to include C-terminal cysteine, MW 34.3kD; thio-ether coupling; no animal-derived components in manufacturing.

<sup>c</sup>Recombinant Protein A coupled through multipoint attachment; no animal-derived components in manufacturing.

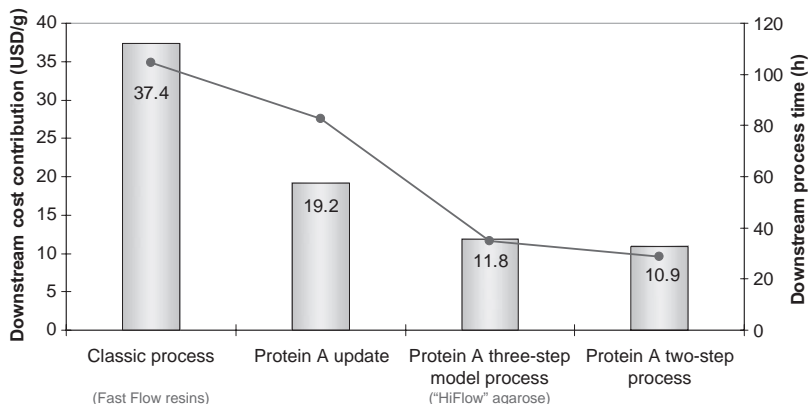
<sup>d</sup>Tetrameric, engineered domains (B only) (asparagine-glycine replacement) to confer alkali stability; MW = 26.7kDa; thio-ether coupling through C-terminal cysteine; no animal-derived components in manufacturing.

<sup>e</sup>Replaced ProSep A High Capacity; no animal-derived components in manufacturing.

<sup>f</sup>Reduction in pore size provides higher surface area and thus 30%–50% higher capacity; no animal-derived components in manufacturing.

<sup>g</sup>POROS products use Protein A (domains E, D, A, B, C; MW 45kDa) from Repligen Corp. Repligen does not use animal-derived components in manufacturing.

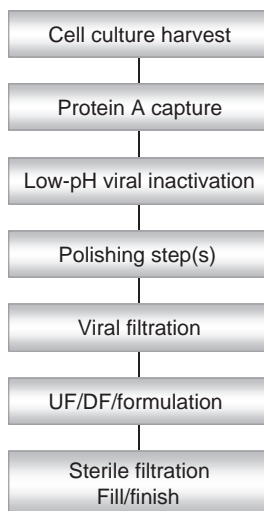




**FIGURE 2.2** Before alternatives were introduced, classic antibody processes were developed on native or recombinant full-length Protein A immobilized on Fast Flow agarose resins. Molecular engineering of Protein A led to significant improvements of about 48% reduction in cost contribution from the affinity resin, and introduction of more rigid matrices led to a further 38% drop. Further contributions are possible with the introduction of a two-step process. Importantly, processing time has been reduced from about 100h in the classic process to less than 2 days. The cost of using Protein A resins has dropped from over US\$35 per gram of antibody produced to about US\$10 (131). Reproduced courtesy of G. Jagschies, GE Healthcare Bio-Sciences AB.

product by the host organism. The now classic platform technology for mAb purification (84), shown in Fig. 2.3, contains chromatographic polishing steps that target not only HCP and DNA reduction but also high-molecular-weight (HMW) aggregates, low-molecular-weight (LMW) degradation products, and leached Protein A. The use of the synthetic dye ligand Cibacron Blue in flow-through mode has been shown to reduce levels of bovine serum albumin, HCP, and even unwanted product variants (85), but it is probably not used in commercial antibody manufacturing, added to which manufacturers strive to eliminate animal-derived products from processing. Specific technologies such as nanofiltration and low-pH treatment are used to remove and/or to inactivate viruses in addition to the partitioning effects of chromatography when the product is bound to the adsorbent (see Chapter 16).

The preferred steps use CEX as a further bind-and-elute operation to reduce HCP, DNA, and aggregates, and AEX in flow-through mode to reduce the levels of HCP and DNA further but also to remove leached Protein A and endotoxins. HIC and hydroxyapatite chromatographic steps may also be used, but newer mixed-mode resins such as Capto™ adhere (86), which are designed specifically to remove product impurities and process contaminants, promise to reduce the purification train to one capture step and one flow-through step performed either by fixed-bed or membrane chromatography (87). The steps



**FIGURE 2.3** Generalized platform for mAb purification. Cell culture harvest employs centrifugation and filtration trains to remove cells and to provide a clarified filtrate for antibody capture on a Protein A adsorbent. Polishing steps are selected from cation (bind-and-elute) and AEX (flow-through) fixed-bed or membrane chromatography, HIC, and hydroxyapatite chromatography. One or both steps may be necessary. Nano-filtration down to <20nm has become a standard viral removal step prior to UF/DF into the formulation buffer followed by sterile filtration and fill/finish operations. These last operations are frequently performed by specialist contractors.

described here constitute the generalized or generic platform for antibody purification prevalent in 2007, as shown in Fig. 2.3. Generic processes have been discussed from a process engineering rather than from a biochemical point of view by Sommerfeld and Strube (88).

Two-thirds of all purification processes for commercial monoclonal antibodies commence with a Protein A capture step. However, notable exceptions include Zenapax<sup>®</sup> (Daclizumab) and Humira<sup>®</sup> (Adalimumab), both of which use CEX capture and AEX flow-through steps albeit in different orders, demonstrating alternatives to Protein A capture as well as downstream process design flexibility. The high price of Protein A has driven many researchers to investigate alternative methods. Follman and Fahrner (89) carried out factorial screening to investigate three-step processes, finding that the step order has significant impact on the clearance of HCP. Three nonaffinity, IEX-based processes using CEX capture were identified (also see Chapter 13).

Arunakumari and colleagues (90) have recently compared different IEX-based process scenarios. They put forward a process design involving CEX capture at a dynamic binding capacity of 100 g/L resin and the elimination of intermediate tangential flow filtration (TFF) prior to flow-through polishing using a Q membrane device (AEX) at an equivalent loading of 2100 g/L

membrane volume. In the move toward very large-scale antibody manufacturing, van Reis has suggested that membrane chromatography solutions (see Chapter 11) could replace fixed-bed operations, and therefore eliminate column packing. Onerous validation could be reduced by the use of disposable cartridge technology. A CEX membrane with a dynamic binding capacity at 1% breakthrough of approximately 28 g/L membrane volume could be followed by an AEX flow-through membrane with heat and low pH as virus inactivation steps in addition to filtration (91). A flow-through Q membrane chromatography step with a loading capacity of 3000 g/m<sup>2</sup> has been evaluated in detail (92).

It comes as no surprise that membrane chromatography has now found its place in antibody processes as a means for impurity and contaminant removal rather than for bulk adsorption operations. The concepts were first described as the “chromatopile” stack of filter papers by Mitchell and colleagues in 1949 (93). Modern membrane chromatography combines the convective flow/low-pressure advantage of membranes with their mass transfer capacity and has led initially to stacked membrane solutions. The development of the first membrane stacks (2–50 membranes) by Brandt and colleagues in 1988 (94) can be viewed as the equivalent of shortening the column to near zero length, allowing large-scale processes to run with only a small drop in pressure even at high flow rates, and therefore resulting in higher productivity. These devices were also applied to the separation of immunoglobulins from plasma (95). At the same time, “radial-flow chromatography cartridges” in a spiral winding format became available and were also used to purify immunoglobulins (96). Somewhat earlier, Jungbauer had used what he described as “radial streaming ion exchange chromatography” in the purification of mAbs, using Q membrane cartridges to bind the main protein impurities from mouse ascites preparations (97, 98). Adapting the technique of thiophilic chromatography introduced by Porath and colleagues (99), thiophilic membranes were also developed for the capture of mAbs from cell culture supernatants (100). However, only the Q (or DEAE) fixed-bed IEX polishing step has been replaced by membrane chromatography in commercial processes (92).

In addition to the various biochemical engineering approaches to improve separations achieved by displacement chromatography, as well as innovative ligand technology, improved bead matrices, monolithic structures, and membrane chromatography, there are also new developments in systems engineering that are likely to affect antibody purification processes. These include radial-flow chromatography in fixed beds (101), multiple-column chromatography [an adaptation of simulated moving bed (SMB) technology to biopharmaceutical processing; see Chapter 18] (102), and expanded-bed engineering. A cost model for Protein A capture at 1 and 5 g/L titers from bioreactors up to 20,000 L in capacity has been developed showing very significant cost savings, mainly in consumables (Protein A adsorbent), of which about 50% are feasible (A. Sinclair, pers. comm.). Early results from small-scale laboratory experiments show that a 27% increase in effective capacity utilization can

be achieved on a 12-column, 60-mL total bed volume system compared to a conventional batch, single 135-mL column (103). In addition, capture efficiency was improved from 99% to 99.5% and HCP reduction was increased from a log reduction value (LRV) of 2.2 to 2.6.

“Process chromatography has the notoriety of being the single largest cost centre in downstream processing” and Przybycien and colleagues (104) have asked if there is “life beyond packed bed chromatography?” Among the current chromatographic alternatives, SMB with its small column number (<12) is most likely to make a major impact on downstream processing. Alternatives to chromatography have been reviewed by Thömmes and Etzel (105), who focus attention on membrane chromatography, precipitation, crystallization, high-resolution ultrafiltration (UF) and high-pressure refolding (see Chapters 8–10).

Precipitation, long used in the polyclonal antibody industry, is under investigation as a possible substitute for Protein A capture (106) (see Chapter 9). Although different antibodies behave somewhat differently, step recoveries are high (>95%) and biochemical, biophysical, and biological assays suggest purity comparable to a conventional Protein A capture step (107). Initial studies indicate higher HCP levels than in nonoptimized systems.

## **2.5 PURIFICATION OF mAbs APPROVED IN NORTH AMERICA AND IN EUROPE**

Procedures for polyclonal antibodies may not be directly applicable to mAb purification because of the very different impurity profiles of the starting feed streams, but there are areas of commonality such as the methods of virus inactivation and removal. The global production of IVIG was 80 tons in 2007 (108) and is projected to reach approximately 100 tons by 2010, with six manufacturers accounting for the bulk of the output. In addition, with a market sales price of circa \$70 per gram (109), there is immense pressure on polyclonal manufacturing efficiency and supply chain management. As the output of mAbs from the major owners of cell culture capacity continues to move toward the 1-ton level, the lessons learned in polyclonal antibody manufacturing become more relevant since these processes use conventional chromatographic and membrane unit operations in the processes downstream of the Cohn precipitates (110). The feasibility of operating a 10-ton mAb process is also under consideration (111), but such processes would still be significantly smaller than the annual output of polyclonal antibodies from the major blood plasma fractionators.

In 1991, Ransohoff and Levine (112) discussed the methods available at that time appropriate for commercial antibody manufacturing. The authors addressed the complete palate of chromatographic technologies, identifying issues with Protein A that have subsequently been addressed through molecular engineering and immobilization developments. Later, Birch and

colleagues (113) in a review of production technologies also addressed the pros and cons of the methods as well as possible sequences, noting in 1995 that cell culture could be scaled to 10,000 L. Financial performance, healthcare cost pressures in the last decade, and biotechnology industrialization have contributed to the reassessment of manufacturing technologies. During the same period, cell culture titers have increased, significantly putting pressure on the throughput of downstream processes (114). It is not the shortcomings of current downstream processing unit operations that cause the so-called bottleneck (115) but the improvement of cell culture titers equivalent to the antibody level in ascites (5–20 g/L) or the expression levels achieved in transgenic caprine and ovine mammary glands. The titer of Humira in Chinese Hamster Ovary (CHO) cells has reached 6 g/L (116), and to cope with 40-kg bioreactor batches and a product output of 1000 kg/year, Sofer and Chirica (117) project high-flow ion exchangers running at over 700 cm/h in 20-cm columns and at capacities of 100 g/L at residence times of 2–6 min. Discussing the future of antibody purification, Low and colleagues (118) conclude that the “true bottleneck in recovery processes is the first adsorptive column.” As discussed by Kelley (111), conventional (meaning current) unit operations—resins, membranes, and equipment—can cope with the output of a 15,000-L bioreactor at a 5 g/L titer.

The “established” processes for mAb manufacturing have been reviewed by Farid (119), who concludes that the unit operations and sequences are very similar (88). This is evident in Tables 2.2–2.6, which focus on the downstream processes for monoclonal antibodies approved in the USA and in Europe. The actual purification sequences may not be in the order given—data were derived from the author’s own notes, prescription information from the products, reference 88 and publicly available regulatory information (120–122).

Of the 29 antibody purification descriptions given, 18 (62%) processes use Protein A as the initial capture step and 3 (10%) use CEX capture on non-agarose-based resins. At least six (20%) processes use both CEX and AEX downstream of the main capture step. Four processes use HIC and only three use size exclusion in the final polishing steps. The use of hydroxyapatite is mentioned in one process. The use of nanofiltration is stated in at least 14 (48%) of the processes but is probably used in the majority as is a low-pH hold step for virus inactivation. Ultrafiltration/diafiltration (UF/DF) is ubiquitous in all described processes. There is a striking similarity in the processes despite the different cell lines used and the different antibody formats, although most products are of the IgG1 $\kappa$  subtype. The appropriateness of a platform approach is emphasized in Table 2.5, which presents data for recombinant, humanized antibodies. However, Shukla and colleagues (84) point out that although many parameters can be fixed, even for the robust Protein A capture step, resin loading capacity and wash and elution buffers need to be optimized for the antibody in question. Furthermore, it should be noted that functional groups, substitution levels, matrix chemistry, and morphology differ widely among the many available IEX resins (123–128) and that screening of

**TABLE 2.2 Purification of Murine Monoclonal Antibodies Produced *In Vivo* from Ascites Fluid**

Product and Company	Indication	US Approval	Antibody Format	Purification
Orthoclone OKT <sup>®</sup> 3/ Muromomab CD3 (Ortho Biotech/J & J) CEA-Scan/Arcitumomab (Immunomedics, Inc.)	Acute kidney transplant rejection  Diagnostic imaging: colorectal cancer	1986  1996	IgG2a  Fab' derived from IgG1, technetium 99-m conjugate	Ammonium sulfate precipitation, AEX chromatography  Q Sepharose, Protein A Sepharose, S Sepharose, pepsin cleavage, chromatography incl. Q Sepharose, size-exclusion HPLC
LeukoScan <sup>®</sup> /Sulesomab (Immunomedics Europe)	Diagnostic imaging: location and extent of infection/ inflammation in bone osteomyelitis	Europe and Canada only	Fab'2 fragment, technetium 99-m conjugate	Q Sepharose, Protein A affinity of IgG, additional chromatography, enzymatic cleavage by pepsin to F(ab') <sub>2</sub> , S Sepharose, chemical conversion to the active substance Fab'-SH and chromatographic purification

**TABLE 2.3 Purification of Murine Monoclonal Antibodies Produced by *In Vitro* Hybridoma Cell Culture**

Product and Company	Indication	US Approval	Antibody Format	Purification
OncoScint® CR/OV/ Satumomab pendetide (Cytogen Corp.)	Diagnostic imaging: ovarian/colorectal carcinoma	1992	IgG1k, indium 111 conjugate	See ProstaScint purification
Verluma/Nofetumomab (NeoRx, now Poniard Pharmaceuticals, Inc.)	Diagnostic imaging: small-cell lung cancer	1994	Fab derived from IgG2b, technetium 99-m conjugate	Papain digestion; chromatographic purification, viral inactivation
ProstaScint®/Capromab pendetide (Cytogen Corp.)	Diagnostic imaging: prostate cancer	1996	IgG1k, indium 111 conjugate	Sephadex G-25, Protein A Sephacrose, DEAE-Sephacrose, S Sephacrose
Zevalin®/Ibritumomab tiuxetan (Biogen Idec)	Radioimmunotherapy in non-Hodgkins lymphoma	2002	IgG1k, indium 111 and yttrium Y-90 conjugates	ProSep A, UF/DF, AEX, HIC, nanofiltration, UF/DF
Bexxar® (Tositumomab) (Corixa, now GlaxoSmithKline)	Radioimmunotherapy in non-Hodgkins lymphoma	2003	IgG2a, iodine I131 conjugate	Not available

**TABLE 2.4 Purification of Recombinant Chimeric (Murine-Human) Monoclonal Antibodies from *In Vitro* Cell Culture**

Product	Indication	US Approval	Antibody Format	Purification
ReoPro <sup>®</sup> /Abciximab (Centocor, Inc./Eli Lilly)	Prevention of thrombus formation in PCI	1994	Fab fragment in SP2/0 murine myeloma	Chromatography, papain digestion, viral inactivation
Rituxan/MabThera <sup>®</sup> / Rituximab (Biogen Idec/ Genentech, Inc.)	Non-Hodgkins lymphoma, rheumatoid arthritis	1997	IgG1κ in CHO	Protein A chromatography, UF/ DF, AEX, Q Sepharose, nanofiltration
Remicade <sup>®</sup> /Infliximab (Centocor/J&J)	Rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, plaque psoriasis, ulcerative colitis	1998	IgG1κ in SP2/0 murine myeloma	Protein A Sepharose, UF, CEX, S/D (TNBP/Polysorbate 80) viral inactivation, virus filtration, primary AEX, secondary AEX, UF
Simulect <sup>®</sup> /Basiliximab (Novartis)	Prophylaxis of acute organ rejection in de novo renal transplantation	1998	IgG2a in murine myeloma	Eight purification steps including two viral removal/inactivation steps: IEX capture, Protein A chromatography, two further IEX steps
Erbix <sup>®</sup> /Cetuximab (Imclone Systems/ Bristol-Myers Squibb)	Colorectal cancer	2004	IgG1 in murine myeloma	POROS Protein A capture, Q Sepharose, viral filtration, S Sepharose, UF/DF



**TABLE 2.5 Purification of Recombinant Humanized Monoclonal Antibodies from *In Vitro* Cell Culture**

Product	Indication	US Approval	Antibody Format	Purification
Zenapax/Daclizumab (Protein Design Labs/Hoffman-La Roche)	Prophylaxis of acute organ rejection in renal transplantation	1997	IgG2a in GS-NS0	Q Sepharose chromatography (flow-through), S Sepharose chromatography, pH treatment for viral inactivation (pH 3.6–3.8, 30–35 min), concentration/diafiltration (DF), DV50 filtration for virus removal, Q Sepharose II chromatography (flow-through), Viresolve filtration, concentration by UF, S-300 gel filtration chromatography, UF
Synagis®/Palivizumab (Medimmune)	Prevention of respiratory syncytial virus (RSV) lung infections in children	1998	IgG1 in GS-NS0	POROS HS 50 (CEX capture), benzonase treatment, Protein A, nanofiltration, low-pH hold, Super Q, UF/DF
Herceptin®/Trastuzumab (Genentech Inc./Roche)	Metastatic breast cancer	1998	IgG1 in CHO	Protein A chromatography, incubation at low pH (<3.7), CEX chromatography, AEX chromatography, and HIC
Enbrel®/Etanercept (Immunex Corp., now Amgen, Inc.)	Rheumatoid arthritis, psoriatic arthritis, plaque psoriasis, ankylosing spondylitis	1998	Dimeric fusion protein: TNF receptor + IgG1 Fc in CHO	Protein A chromatography, UF, AEX, Ceramic hydroxyapatite elevated temperature hold viral filtration, UF/DF
Mylotarg®/Gentuzumab (Wyeth Pharmaceuticals, Inc.)	Acute myeloid leukemia	2000	IgG4κ In GS-NS0	DEAE-Sepharose, low pH, viral filtration
Campath-1H®/Alemtuzumab (Millenium/Berlex)	B-cell chronic lymphocytic leukemia	2001	IgG1κ in CHO	Protein A capture, Sartobind Q, S Sepharose, nanofiltration [DV50, UF/DF (30K membrane)], Superdex 200 gel filtration

Xolair®/Omalizumab (Genentech, Inc.)	Persistent asthma	2003	IgG1κ in CHO	Protein A affinity capture, CEX chromatography (SP Sepharose), AEX chromatography (Q Sepharose), UF/DF Chromatography, viral and UF/DF
Raptiva®/Efalizumab (Genentech, Inc.)	Severe plaque psoriasis	2003	IgG1 in CHO	Protein A affinity capture, ceramic hydroxyapatite, AEX, viral filtration
Amevive®/Alefacept (Biogen Idec)	Moderate to severe chronic plaque psoriasis		Dimeric fusion protein, LFA3 + IgG1 Fc in CHO	Protein A chromatography, flow-through AEX chromatography (Q Sepharose FF), CEX chromatography (CM Sepharose FF), UF/DF
Avastin®/Bevacizumab (Genentech, Inc.)	Metastatic rectal and colon cancer	2004	IgG1 in CHO	Protein A Sepharose, AEX chromatography, HIC, UF/DF, nanofiltration
Tysabri®/Natalizumab (Biogen Idec)	Multiple sclerosis	2004	IgG4κ in NS0	Four chromatographic steps incl. Protein A capture, viral inactivation, nanofiltration, UF/DF
Orencia®/Abatacept (Bristol-Myers Squibb)	Rheumatoid arthritis	2005	Dimeric fusion protein, CTLA- 4 + IgG1 Fc in CHO	CEX chromatography (POROS HS 50), UF/DF, low-pH treatment, nanofiltration
Soliris®/Eculizumab (Alexion Pharmaceuticals, Inc.)	Paroxysmal nocturnal hemoglobinuria	2007	IgG2/4κ in NS0	

**TABLE 2.6 Purification of Fully Humanized Monoclonal Antibodies from *In Vitro* Cell Culture**

Product	Indication	US Approval	Antibody Format	Purification
Humira/ Adalimumab (Abbott Laboratories)	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease	2002	IgG1 in CHO	Fractogel S (CEX capture), Q Sephacrose, Phenyl Sephacrose, low-pH viral inactivation
Vectibix™/ Panitumumab (Amgen, Inc.)	Metastatic colorectal cancer	2006	IgG2κ in CHO	Protein A chromatography, low-pH viral inactivation, polishing chromatography steps, UF/DF

resin candidates should therefore be carried out for optimal process development (129). The use of IEX membranes in flow-through mode operations in preference to fixed column operation has already been discussed, but attention needs to be paid to virus and HCP clearance in the choice of membrane.

In Tables 2.2–2.5, the antibodies are grouped by source—ascites, hybridoma cell culture, recombinant antibodies, and finally, fully humanized antibodies, which, together with the approval dates (in the USA), demonstrate the development of processes from ammonium sulfate precipitation and AEX to the two alternative capture platforms of Protein A or strong cation exchangers. The state of the art in 2007 is to follow the capture step with one or, at most, two polishing steps integrated with minimal UF/DF steps in between the unit operations and virus inactivation and removal by nanofiltration to enhance product purity and safety.

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## HARVEST AND RECOVERY OF MONOCLONAL ANTIBODIES: CELL REMOVAL AND CLARIFICATION

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### 3.1 INTRODUCTION

Monoclonal antibodies (mAbs) occupy an important niche within the biopharmaceutical industry (1). As shown in Table 3.1, many of these molecules are now commercially approved, and they are used to treat a wide range of clinical indications. In addition, a significant proportion of drug candidates in clinical development are mAbs, indicating that the importance of this therapeutic modality will continue to grow (2). The widespread use of mAbs reflects their ability to target a wide range of cellular receptors and cytokines, either to block or to augment their function, to induce their destruction through antibody-dependent cell cytotoxicity (ADCC), or to activate the complement cascade (3).

Until recently, all commercialized mAbs were produced in mammalian cell culture systems. This trend dates back to the initial creation of mAbs in 1975 by Kohler and Milstein (4), who fused a B-lymphocyte with an immortal mammalian cell line to create the first hybridoma. While hybridomas are rarely used for commercial mAb production, a variety of other mammalian cell culture systems have been developed, including Chinese hamster ovary (CHO), SP 2/0, and NS0 cells (5–7). Mammalian cells are favored because of the complexity and the large size of mAbs, which comprise two heavy and two light chains joined together by covalent and noncovalent bonds to achieve

**TABLE 3.1 Approved mAb Therapeutics**

Trade Name	Target	Indication	Company	Approval Year	Antibody Type
Orthoclone OKT3	CD3	Acute kidney transplant rejection	Ortho Biotech	1986	Murine
ReoPro	Platelet GP IIb/IIIa	Prevention of blood clot	Centocor	1994	Murine
Rituxan	CD20	Non-Hodgkin's lymphoma	Genentech/Biogen-IDEC	1997	Chimeric
Panorex	17A-1	Colorectal cancer	GlaxoSmithKline	1995	Murine
Zenapax	IL2R $\alpha$ (CD25)	Acute kidney transplant rejection	Hoffman-LaRoche	1997	Humanized
Simulect	IL2R	Prophylaxis of acute organ rejection in allogeneic renal transplantation	Novartis	1998	Chimeric
Synagis	RSV	Respiratory syncytial virus	Medimmune	1998	Humanized
Remicade	TNF $\alpha$	Rheumatoid arthritis	Centocor	1998	Chimeric
Herceptin	Her2/neu/ErB2	Metastatic breast cancer	Genentech	1998	Humanized
Mylotarg	CD33	Acute myelogenous lymphoma	Wyeth-Ayerst	2000	Humanized
Campath-1H	CD52	B-cell chronic lymphocytic leukemia	Millenium/ILEX	2001	Humanized
Zevalin	CD20	Non-Hodgkin's lymphoma	Biogen IDEC	2002	Murine
Humira	TNF $\alpha$	Rheumatoid arthritis	Abbott	2002	Human
Bexxar	CD20	Non-Hodgkin's lymphoma	Corixa/GSK	2003	Murine
Xolair	IgE	Allergy	Genentech/Novartis	2003	Humanized
Erbitux	EGFR/Her1	Colorectal cancer	Imclone/Bristol-Myers Squibb	2004	Humanized
Avastin	VEGF	Metastatic colon cancer	Genentech	2004	Humanized
Raptiva	CD11a	Psoriasis	Genentech/Xoma	2004	Humanized
Tysabri	$\alpha$ -4 Integrin	Multiple sclerosis	Biogen/Idec	2004	Humanized
Vectibix	EGFR	Colorectal cancer	Amgen	2006	Human
Soliris	C5 complement	Paroxysmal nocturnal hemoglobinuria (PNH)	Alexion	2007	Humanized

the well-known Y-shaped conformation. Furthermore, mAbs are glycoproteins whose glycan chains could play a significant role in their biological activity, and only mammalian cells produce the correct glycan chain structures (8). In recent years, transgenic sources (both animals and plants) have been developed for the production of clinical-stage mAbs (9). While these expression systems can certainly produce mAbs, concerns about prion diseases in transgenic animals have held up the widespread adoption of transgenic animal production systems. Additionally, concerns about the potential immunogenicity of plant glycans, together with societal concerns over transgenic plants, have restricted the application of transgenic plant production technologies. Very recently, mAbs have been successfully expressed in *Escherichia coli* (10). The lower costs of bacterial expression could favor the further adoption of this expression system in the future. Given the current commercial preponderance of mammalian expression systems, this chapter will focus on the harvest of mAbs from large-scale mammalian cell cultures.

Extracellular expression in mammalian cell culture provides both opportunities and restrictions for the design of robust harvest and clarification operations. With advances in cell culture media and cell lines, it is now possible to achieve high titers of over 5 g/L for mAbs, thus reducing batch harvest volumes. The high cell densities achieved in these systems (1–6 g/L) are still lower than those seen in yeast or bacterial fermentation systems. However, mammalian cells are sensitive to breakage due to shear stress, and this can result in the release of proteases and other host cell proteins (HCPs) into the broth, which can affect product stability and/or purity. Cell culture media are fairly rich and can promote bacterial growth in the event of a contamination. As a result, hold times for both the production bioreactor after the termination of cell culture and for the harvest operations need to be limited.

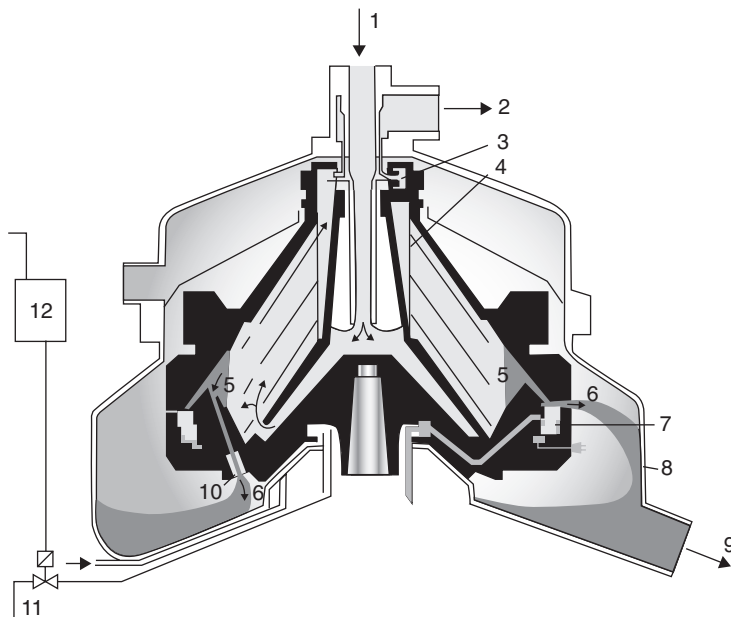
The large number of mAbs undergoing clinical development has placed significant pressure on process development organizations to harmonize efforts through the adoption of platform technologies (11). These efforts facilitate the use of one manufacturing facility to produce multiple products as well as streamline the documentation required for current good manufacturing practice (cGMP) production. In the same spirit, most companies have also started to adopt platform harvest and clarification technologies.

Several unit operations have been employed for cell removal and clarification during the processing of products secreted from mammalian cells, most of which have been comprehensively reviewed in the context of product recovery from yeast expression systems that employ similar unit operations (12). The first harvesting step is to remove the cells followed by filtration to provide additional clarification. Centrifugation and microfiltration are the primary harvest techniques used in the industry. While depth filtration can be employed as the harvest method, it is more common to see this unit operation follow the harvest step to provide additional clarification. Flocculants are sometimes added prior to harvest to augment the harvest and clarification operations. Filtration through absolute pore size membranes is typically the final step in

harvest and clarification sequence prior to capture chromatography. Expanded-bed chromatography has been developed as an integrated unit operation that combines harvest with product capture. This chapter discusses each of these unit operations in further detail and aims to provide the reader with a basic understanding of the fundamentals as well as the practical considerations during process development. The final section describes how these unit operations come together in an industrial context to form the harvest and clarification process for mAb products. This chapter provides a broad review of these harvest and clarification techniques in the hope that this will be useful for those entering the field. For those already engaged in this area, we hope the references will be a valuable resource for further in-depth reading.

### 3.2 CENTRIFUGATION

Centrifugation exploits the density difference between solids and the surrounding fluid. The centrifugal force accelerates the settling that would normally occur during sedimentation. Most industrial applications utilize disk-stack centrifuges to remove cells and cell debris (13). Disk-stack centrifuges operate continuously, making their throughput consistent with the desire to limit the time for harvest operations. Figure 3.1 shows the schematic for a disk-stack centrifuge.



**FIGURE 3.1** Schematic of a disk-stack centrifuge (courtesy of Westfalia Inc.). (1) Product feed, (2) clarified liquid discharge, (3) centripetal pump, (4) disks, (5) solids space, (6) solids discharge, (7) desludging mechanism, (8) concentrate catcher, (9) concentrate outlet, (10) nozzles, (11) operating water feed, (12) timing unit.

The basic principles of centrifugation involve a balance between the buoyant force acting on solid particles and Stokes' law, which expresses the drag force. For a tubular bowl centrifuge, the flow rate over the surface area used for settling can be expressed as

$$\frac{Q}{A} = \frac{d^2 (\rho_s - \rho_L)}{18\mu} g \quad (3.1)$$

where  $\rho_s$  and  $\rho_L$  are the density of the solid and liquid, respectively;  $d$  is the diameter of the particle;  $\mu$  is the viscosity of the liquid, and  $g$  is the acceleration due to gravity. The equations for a disk-stack centrifuge have been developed in an analogous fashion to determine the ratio of the flow rate to the equivalent surface area for settling (14). For a disk-stack centrifuge

$$\Sigma_{\text{DSC}} = \frac{2\pi n \omega^2 (r_o^3 - r_i^3)}{3g \tan \theta} \quad (3.2)$$

where  $n$  is the number of disks;  $r_o$  and  $r_i$  are the outer and inner radii of the disk, and  $\theta$  is the angle of the disks from the vertical. This equation has recently been modified to account for the acceleration and deceleration phases of the operation (15). During the scale-up of a centrifugation operation, the ratio of flow rates to the effective settling area is held constant. This is particularly useful if operating conditions are first screened at the laboratory scale in a tubular bowl centrifuge and are then employed to decide on the operating conditions for the disk-stack centrifuge.

The operating conditions (flow rate, revolutions per minute) for the disk-stack centrifuge still require some empirical optimization even once the appropriate  $\Sigma$  has been determined. This reflects the certain degree of cell disruption that occurs due to shear stress during centrifugation, which generates smaller particles that cannot be removed efficiently by the centrifuge under the operating conditions based on the following equation (16):

$$\frac{Q_1}{\Sigma_1} = \frac{Q_2}{\Sigma_2} \quad (3.3)$$

In general, long residence times (slow flow rates) will lead to a clearer centrate but at the expense of process throughput. The clarification efficiency of a centrifuge can be determined by the measurement of relative turbidity in the feedstream and the centrate. Not all aspects of a disk-stack centrifuge can be captured effectively by a laboratory-scale tubular bowl centrifuge. In particular, shear stresses during entry and discharge from the bowl are difficult to scale. A scaled-down version of a disk-stack centrifuge that requires less than 10 L of broth to operate has been developed to accelerate the experimental development of operating conditions (17).

Other operating parameters that require optimization for a continuous disk-stack centrifuge operation include the discharge frequency, the discharge

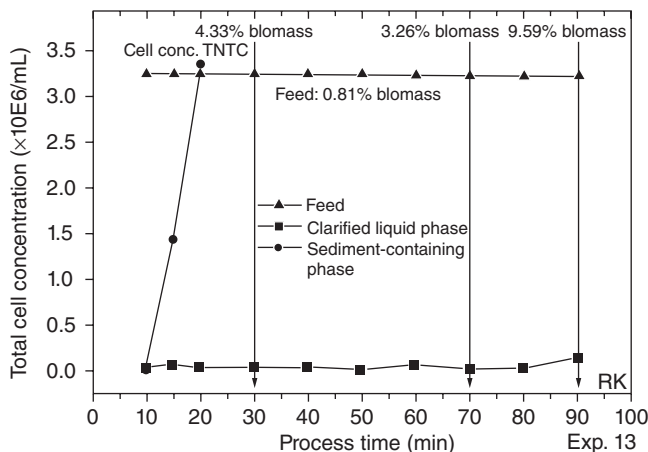


type and the predischARGE flush solution and volume. These parameters are determined empirically. The effectiveness of a centrifuge in removing particulates decreases as the bowl fills with the deposited solid sludge. However, discharges at too frequent intervals will risk reducing the product yield and increasing the operating time. A practical compromise is to discharge the bowl after it fills to 50%–70% of the bowl volume. The discharge frequency is determined by the solids content of the cell culture broth. Experimentation with the ideal case of continuous discharge in a nozzle centrifuge (18) is ongoing for harvested mammalian cell culture media, but has yet to be used in a large-scale operation. In the case of discontinuous bowl discharge, the discharge type can be full or partial depending on whether or not the entire bowl contents are ejected during the discharge. Prior to discharge, a flush volume is used to push the contents of the bowl through the centrifuge so that yield losses during discharge are minimized. Buffer or water can be used for the flush. An important consideration is whether the osmotic difference between the flush fluid and the cell culture broth will cause cell lysis, releasing cell debris, HCP, DNA, and proteases, all of which can reduce product quality. Flushing is more commonly carried out before full shots, and in the case of partial bowl shots, no separate flush liquid tends to be used.

The importance of optimizing the bowl discharge procedure can be illustrated effectively by a case study for the harvest of hybridoma cell culture broths (19). In this study, the use of an intermittent discharge technique led to the disruption of ~20% of the cells that stayed in the bowl. A continuous discharge strategy was therefore investigated. Continuous sediment discharges were used to reduce the residence time of cells in the bowl from 30 min to approximately 1–2 min. However, even here the operation was found to be highly sensitive to the cell densities that were discharged. At low cell densities in the feedstock, the cell sediment accumulated in the sediment holding space and was discharged when some packed sediment was mobilized by chance or by pressure fluctuations (Fig. 3.2). In contrast, the disk-stack centrifuge did not cope well with cell discharge at very high cell densities (data not shown). Significant fluctuation of pressure and fluid flow occurred with this mode of operation. The same amounts of cells were destroyed inside the centrifuge as reported for the intermittent discharge system. Hence, the continuous discharge system operated well only within a narrow range. Even though product recovery was maintained in all these experiments, additional clarification/filtration steps were necessary to remove cell debris and particles prior to downstream processing of the harvested liquid.

### 3.3 MICROFILTRATION

Tangential flow (cross-flow) microfiltration is as popular as centrifugation for the harvest of therapeutic products from mammalian cell cultures (20, 21). One advantage this technique offers is the creation of a particle-free harvest stream



**FIGURE 3.2** Continuous discharge from a disk-stack centrifuge at low cell densities for hybridoma cell culture broth (19).

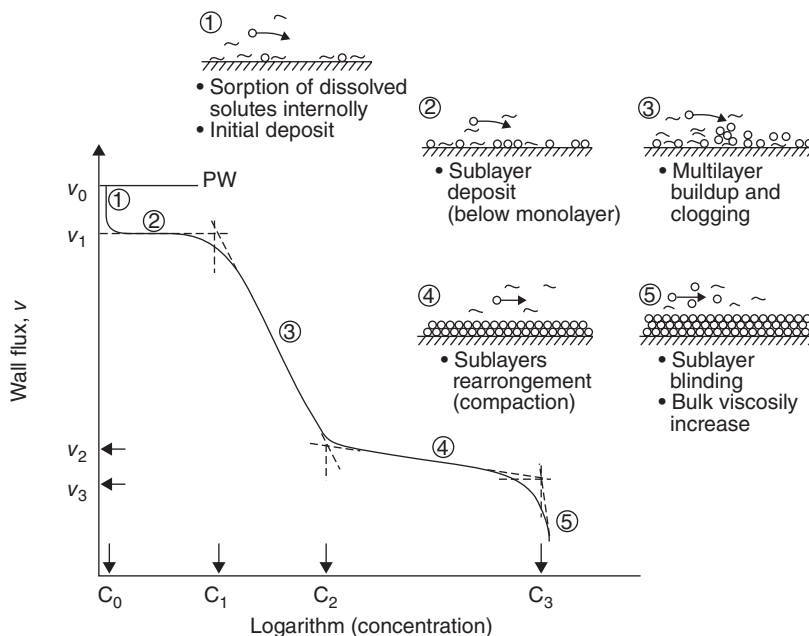
that requires minimal additional filtration. Microfiltration membranes exist with a variety of pore sizes ranging from 0.2 to 0.45  $\mu\text{m}$ . Newer asymmetric membranes with graded pore sizes have significantly improved the throughput of microfiltration harvest operations and have minimized the effect of concentration polarization.

Mass transport limitations due to the formation of a concentration polarization layer of particles close to the membrane surface remain a significant limitation of microfiltration harvest operations (22). Irreversible membrane fouling by particulates in the cell culture broth is another significant concern. A variety of alternative flow configurations have been proposed to mitigate the effects of concentration polarization. These include the use of rotating disk filters that augment the cross-flow velocity close to the membrane surface, thus sweeping the concentration polarization layer away (23), the use of Taylor vortices (24), and the use of Dean vortices created by flow patterns within the microfiltration device, reducing concentration polarization without any moving parts in the microfiltration system (25). Yet another strategy has been the use of periodic backflushing to sweep the membrane surface clean (26).

The gel polarization model describes the relationship between flux and concentration in the mass transport-controlled regime for cross-flow microfiltration. The solute travels to the membrane by convective transport and returns to the liquid phase by diffusion, as described:

$$J = \frac{D}{\partial} \ln \frac{C_g}{C_b} \quad (3.4)$$

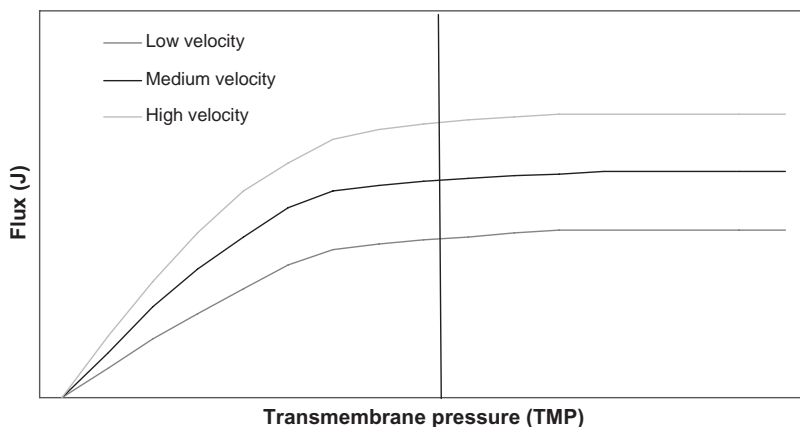
where  $J$  is the flux;  $D$  is the diffusion coefficient;  $\partial$  is the thickness of the gel layer;  $C_g$  is the concentration in the gel layer, and  $C_b$  is the concentration in the bulk phase.



**FIGURE 3.3** Flux decline in microfiltration as explained by different physical phenomena (28).

A comprehensive review of the fundamentals of cross-flow microfiltration and the phenomena involved during concentration polarization and fouling has been provided by Belfort and colleagues (27). The flux decline during microfiltration (28) (Fig. 3.3) has been described in terms of a series of physical phenomena as follows: (i) fast initial buildup of solutes on the membrane surface resulting in rapid but limited flux decline; (ii) buildup of the first layer of suspended cells on the membrane surface during which no flux decline is observed; (iii) buildup of multiple sublayers of cells on the membrane surface resulting in a linear decline in flux against log (concentration) as dictated by the gel polarization model—as several layers build up, there is a decrease in the cross-sectional area for axial flow, thus increasing the wall shear rate and causing back diffusion of the solids into the liquid stream; this effect has been attributed to Brownian diffusion (29) and inertial lift (27); (iv) densification of sublayers during which a slower decline in flux vs. concentration occurs; and (v) increase in bulk viscosity into the non-Newtonian regime resulting in a precipitous flux decline.

The optimal operation of tangential flow microfiltration has been investigated by several researchers (20, 21). A typical flux vs. transmembrane pressure (TMP) relationship is shown in Fig. 3.4. In general, this can be typified by two regimes: (i) a pressure-dependent regime, in which an increase in TMP results in an increase in flux, and (ii) a pressure-independent regime in which



**FIGURE 3.4** Typical flux vs. transmembrane pressure (TMP) profile for cross-flow microfiltration.

increases in TMP do not increase the flux. As a general rule of thumb, it is recommended to carry out the operation at the transition between these regimes to maximize flux while maintaining an acceptable TMP, thus reducing pore plugging and fouling of the membrane. A similar relationship exists for the cross-flow velocity at a given TMP, whose effect also levels off at a certain point. Since TMP and cross-flow velocity are interdependent, one can maintain a constant TMP operation only by manipulating back pressure on the membrane (20) to vary the cross-flow velocity as the operation proceeds and concentration increases.

Microfiltration membranes used for cell culture harvest are often plagued by membrane fouling, i.e., the irrecoverable decline in membrane flux as often measured with clean water. The microfiltration operating conditions and the post-use cleaning process can both help to address this issue significantly. Another important variable is the membrane chemistry, with more hydrophilic membranes generally less susceptible to significant fouling. The decline in flux due to fouling has been detailed by three key models (12).

1. The *pore blockage model*, which assumes that a certain proportion of the pores are blocked completely as filtration proceeds. The flux vs. time relationship in this case is given by

$$\frac{J}{J_0} = \exp\left(-\frac{\alpha_{\text{block}} A J_0 C_b}{N_0} t\right) \quad (3.5)$$

where  $J_0$  is the initial flux;  $\alpha_{\text{block}}$  is the pore blockage efficiency;  $A$  is the membrane surface area;  $C_b$  is the bulk concentration, and  $N_0$  is the initial number of pores.

2. The *pore constriction model*, in which the pore volume is assumed to fall as filtration proceeds. The flux vs. time relationship is given by

$$\frac{J}{J_0} = \left( 1 + \frac{\alpha_{\text{pore}} A J_0 C_b}{\pi r_{\text{pore}}^2 \partial} t \right)^2 \quad (3.6)$$

where  $\alpha_{\text{pore}}$  is the pore constriction coefficient.

3. The *cake filtration model*, which assumes the deposition of a layer (cake) on the membrane surface that then restricts flux in accordance with Darcy's Law.

$$\frac{J}{J_0} = \left( 1 + \frac{2r_c J_0 C_b}{R_m} t \right)^{-0.5} \quad (3.7)$$

where  $R_m$  is the cake resistance and  $r_c$  is the specific resistance of the cake. The cake filtration model has been used to optimize the microfiltration of yeast cells (30).

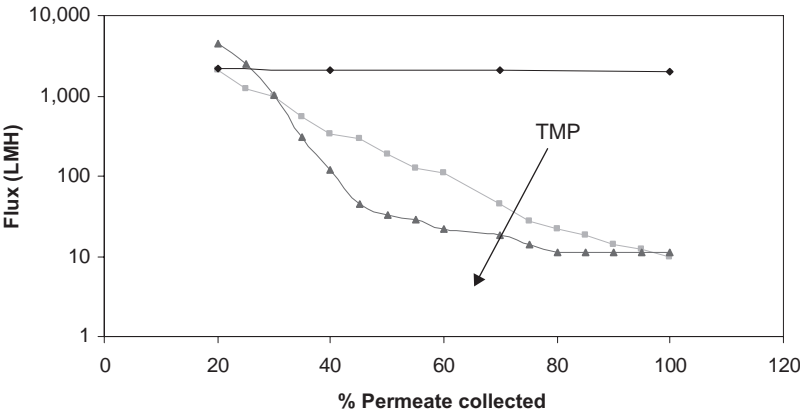
The development of a microfiltration harvest process has been outlined in a step-by-step guide (21) and a brief set of guidelines is presented here. Two important determinants for the use of microfiltration to harvest mammalian cell cultures are the flux and the product yield. The flux determines the surface area of membrane that will be needed to process the cell culture broth, which has significant economic implications. If the flux is too high, the membrane can become fouled, reducing its lifetime, so flux optimization is very important. The selection of the membrane chemistry and pore size are also important for the determination of flux and cleanability. Table 3.2 presents a list of commercially available microfiltration membranes.

The first series of experiments typically involve the measurement of flux vs. TMP and cross-flow rate curves for various concentrations at the laboratory scale. An easy way to carry out these experiments is to operate under total recycle mode, where the permeate is fed back into the load tank to maintain a constant concentration. Steady-state flux can then be measured over different cross-flow velocities to produce the flux vs. TMP plot shown in Fig. 3.4. In these initial experiments, the broth should be concentrated to a degree that represents the final desired concentration. Various membranes can be screened to identify ones that are optimal for the application since both the chemistry and the pore size play an important role in determining flux and flux decay characteristics. It is usual to operate at the transition point between the zones of increasing flux vs. TMP and the zone of TMP-independent flux to maximize flux and to minimize the detrimental effects of fouling and pore plugging.

At this point, further optimization should be carried out at the pilot scale so that the membrane configuration and channel width are representative of

**TABLE 3.2    Commercially Available TFF-MF Membranes for Mammalian Cell Culture Harvest Applications and Their Properties**

Vendor	Membrane	Pore size, $\mu\text{m}$	Description
Millipore	Durapore	0.1, 0.22, 0.45, and 0.65	Open channel hydrophilic PVDF
Millipore	Ultracel PLC	1000 kDa	Composite regenerated cellulose
Millipore	Biomax	1000 kDa	Polyether sulfone (PES)
Pall	Supor	0.2	Modified PES with uniform depth structure
Pall	Omega	650–1000 kDa	PES anisotropic membrane with thin skin layer
Pall	Regen		Regenerated cellulose
Sartorius	Hydrosart	0.2 or 0.45	Stabilized cellulose derivative
Sartorius	PESU	0.1	PES
Sartorius	Polypropylene	0.2	Polypropylene
CUNO	PolyproXL	0.5	Graded density pleated polypropylene
CUNO	BioAssure	0.2	Hydrophilic asymmetric polyethyl sulfone membrane
Pall	Microza hollow-fiber modules	0.8/1.4, 1.4/2.3	PES and polyvinylchloride (PVC)



**FIGURE 3.5    Flux decay profile for microfiltration systems.**

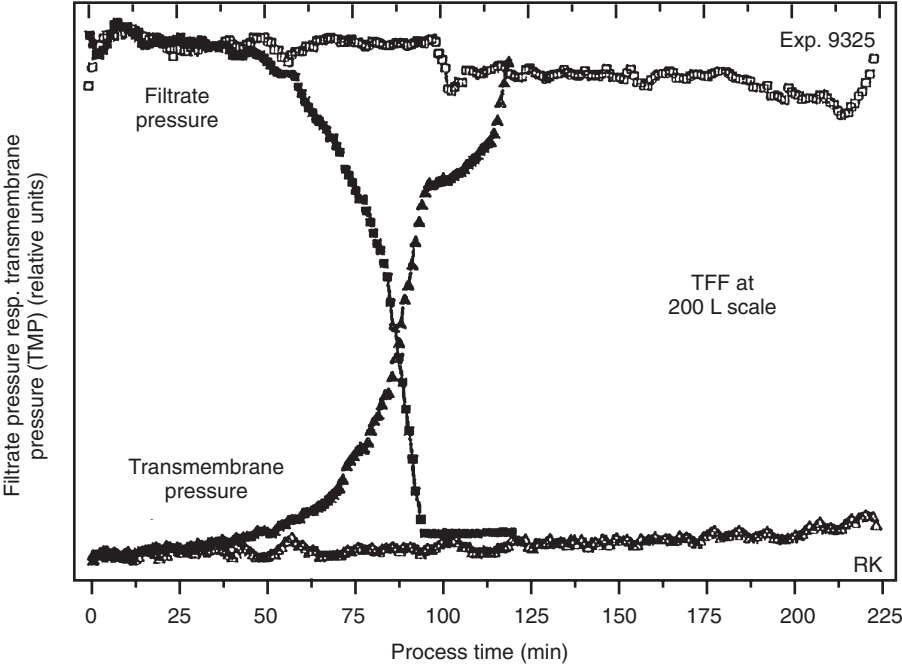
the large-scale operation. Such experiments are carried out under non-steady-state operating conditions (i.e., no recycling) so that concentration varies over the course of the experiment. Membrane loading (i.e., volume of broth processed per unit membrane surface area) is another important parameter for optimization. The experiments should test similar loads to those anticipated in the ultimate industrial process. The flux decay profile, which plots flux vs. time, is one typical measurement taken at this stage (Fig. 3.5). It is often

difficult to predict how TMP will influence flux decay under these conditions from the steady-state experiments conducted earlier—a higher TMP could increase initial flux but could cause more rapid flux decay, or could have a beneficial effect if majority of the total permeate is collected in the very initial stages of the filtration. It is typical to optimize the area under the flux vs. time curve while maintaining an upper limit on the processing time. Cross-flow velocities influence filtration in line with the observations made during the steady-state screening experiments. However, excessively high cross-flow velocities can cause undesirable effects such as cell breakage, which may influence product quality (i.e., HCP and DNA levels) even before a significant impact is seen on the filtration.

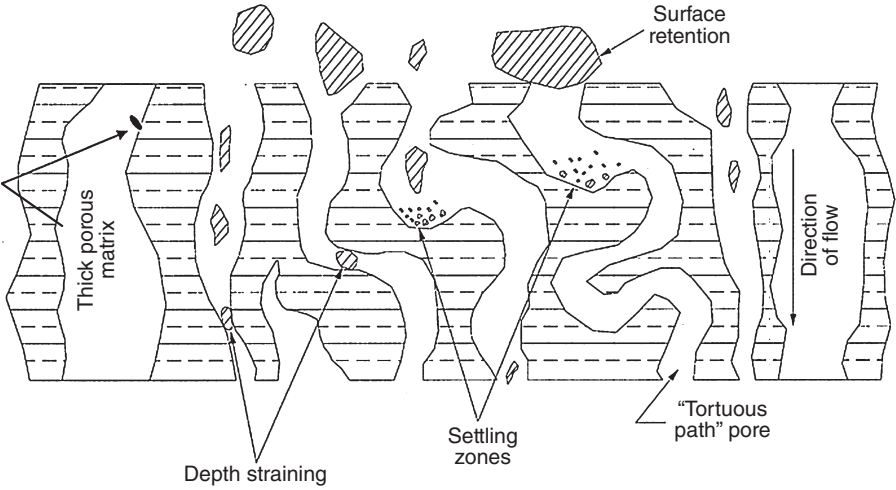
Hollow-fiber microfiltration modules have also been used successfully to harvest mammalian cell cultures (19). The flow rate through the hollow-fiber device can significantly influence its operation, as illustrated in Fig. 3.6, which plots the filtrate pressure and TMP profiles for two pilot-scale experiments. Excessive cross-flow velocity was shown to interfere severely with the outcome of the separation process. At the standard flow rate (120-min process time, solid symbols in Fig. 3.6), rapid membrane fouling was observed, as confirmed by the sharp decline in filtrate pressure and by an increase in TMP. This experiment resulted in a modest product yield of 86%. When the flow rate was halved, the process was considerably longer, but constant filtrate pressure and TMP were maintained (hollow symbols in Fig. 3.6), resulting in a product yield of 98%.

### 3.4 DEPTH FILTRATION

Depth filtration (sometimes called prefiltration or media filtration) refers to the use of a porous medium that is capable of retaining particles from the mobile phase throughout its matrix rather than just on its surface (31). These filters are used when the feedstream contains a higher content of particles (32). In such cases, depth filters can remove larger, insoluble contaminants prior to the final microfiltration step, which would otherwise see the microfiltration membrane clog relatively quickly—hence the term prefiltration (33,34). Depth filters used for bioprocessing are typically composed of a fibrous bed of cellulose or polypropylene fibers along with a filter aid (e.g., diatomaceous earth) and a binder that is used to create flat sheets of filter medium. The filter aid provides a large surface area and is sometimes used alone in clarification applications (35). Some depth filters are charged, either because of the binding polymer or from additional charged polymers (36). Sometimes a microfiltration membrane with an absolute pore size rating is integrated into the depth filter sheet as the bottommost layer. Porous depth filters can retain more particles in their tortuous flow channels than size-based screening alone can achieve, as shown in Fig. 3.7. A variety of depth filters used in biopharmaceutical harvest applications is listed in Table 3.3.



**FIGURE 3.6** Effect of variation of the filtrate flow rate on TFF performance for a hollow-fiber membrane (19).



**FIGURE 3.7** Schematic of depth filter operation in removing particulates.



TABLE 3.3 Depth Filters Applied for Biopharmaceutical Harvest Applications

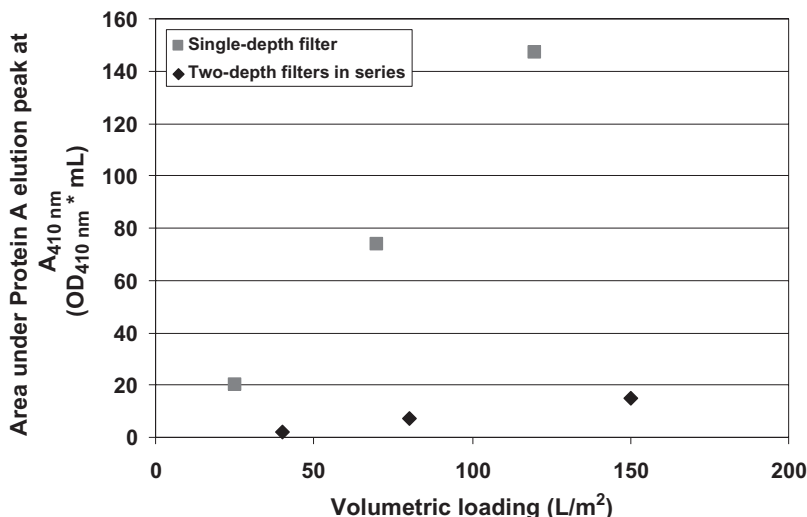
Vendor	Filter	Pore Size, µm	Description
CUNO	10SP	0.8–4.0	Single-layer pharmaceutical-grade media, coarse prefilter
CUNO	90SP	0.2–0.65	Single-layer pharmaceutical-grade media
CUNO	30M03	0.8–4.0 (10SP)/0.6–2.0 (30SP)	Dual-zone construction, high-contaminant holding capacity
CUNO	90M08	0.45–0.8 (60SP)/0.2–0.65 (90SP)	Dual-zone construction, high-contaminant holding capacity
CUNO	120M08	0.45–0.8 (60SP)/0.1–0.45 (90SP)	Dual-zone construction, high-contaminant holding capacity
CUNO	90ZA08A	0.2–0.65	Zeta Plus Maximizer™ EXT series, single-layer inorganic filter aid (DE), high-contaminant holding capacity
CUNO	120ZA10A	0.2–0.65 (90ZA)/0.1–0.45(120ZA)	Zeta Plus Maximizer™ EXT series, dual-layer cellulose fiber combined with inorganic filter aid (DE), high-contaminant holding capacity, positively charged media; upstream zone is more open than the downstream zone
Millipore	Millistak + A1HC	0.1–0.4 (DE65)/<0.1(DE75)	Tightest media combination, two layers of inorganic filter aid (DE) and 0.1-µm nominal cellulosic membrane (RW01)
Millipore	Millistak + B1HC	0.2–0.7 (DE50)/<0.1(DE75)	A more open layer, two layers of inorganic filter aid (DE) and 0.1-µm nominal cellulosic membrane (RW01)
Millipore	Millistak + C0HC	0.2–0.7 (DE30/DE60)	Two layers of an open Millistak+ and DE media
Pall	SupraEK1P	0.2–0.4	P series depth filter; combination of cellulose fibers, DE, and perlite; pyrogen removal capability; positively charged media
Pall	EKSP	0.1–0.3	P series depth filter; combination of cellulose fibers, DE, and perlite; pyrogen removal capability
Pall	Supra 80P	1.5–4.0	P series depth filter; combination of cellulose fibers, DE, and perlite; positively charged media; pyrogen removal capability
Pall	K150	2.5–4.0	K series depth filter; combination of cellulose fibers, DE, and perlite
Pall	SUPRADisc PDD1	0.1–0.85	P series depth filter; two distinct layers; combination of cellulose fibers, DE, and perlite
Pall	SUPRADisc PDD1	0.2–3.5	P series depth filter; two distinct layers; combination of cellulose fibers, DE, and perlite
Sartorius	Sartoclear P S9P	0.1–0.3	Cellulose fibers, DE, and perlite
BEGEROW	PR Steril S 80 UP/PR12 UP	0.2–0.5	Cellulose fibers and inorganic filter aid (DE)
Purolator	NA30KP	0.2–0.4	Cellulose fibers and inorganic filter aid (DE)

For process-scale applications, depth filters are often fabricated into cells consisting of two or more filters separated from each other such that flow occurs from the outside into the space between the layers and is then collected. Multiple cells can be stacked into a housing, and pressure is applied to drive flow through the assembly. Depth filters are usually single-use devices, reducing the amount of process validation required for their inclusion in biopharmaceutical applications.

The large surface area and the possibility of charge-based interactions means that depth filters may have adsorptive properties in addition to their ability to trap larger particles. Positively charged depth filters have been used to remove endotoxins from water (37) and for the removal of virus particles smaller than the effective pore size of the filter as determined by the filtration of small latex beads (38). Positively charged depth filters have also been used to remove DNA from a buffer solution (39). The ability of depth filtration to reduce the DNA content of cell culture harvest fluid was studied more comprehensively for positively charged Zetaplus® 10SP and 90SP depth filters (CUNO, Meriden, CT, USA) (40). Zetaplus VR depth filters removed retrovirus and parvovirus by adsorption when a pure protein solution was spiked with virus (41). More recently, depth filters used to harvest mAbs from mammalian cell culture were shown to reduce HCP impurities that would otherwise precipitate during the subsequent Protein A capture step (42). The filtration operating parameters were optimized to minimize the turbidity observed during Protein A column elution, thus effectively protecting the capture chromatography column. In particular, the total volume of harvest broth loaded per unit depth filter surface area and the number of depth filtration stages in series had the greatest influence on the ability of the depth filter to clear the specific HCPs that caused turbidity during Protein A chromatography (Fig. 3.8). Thus, depth filters not only clarify the broth, but they can also adsorb some otherwise soluble impurities from the feedstream.

In terms of their integration into a harvest and clarification scheme for mammalian cell systems, depth filters are usually placed after a centrifugation step (43). Since centrifugation cannot efficiently remove all particulates from the broth, and microfiltration is too expensive and prone to failure in the event of excessive particle counts from the centrifugation step, a secondary clarification step is usually required, and this niche is filled by depth filtration. Depth filters can be used as the sole harvest and clarification step, but this strategy is only satisfactory at small scale and is neither robust nor cost-effective for large-scale operations. Since most depth filters do not come with an absolute pore size cutoff rating, a dead-ended microfilter is often used in-line after the depth filter for the efficient removal of any residual particulates that might foul downstream chromatographic steps.

During depth filtration process development, it is usual to screen a variety of depth filters with varying chemistries, porosities, and charges. Typical screening experiments are carried out at the laboratory scale to determine the turbidity of the filtrate and any pressure drop increases across the depth filter.



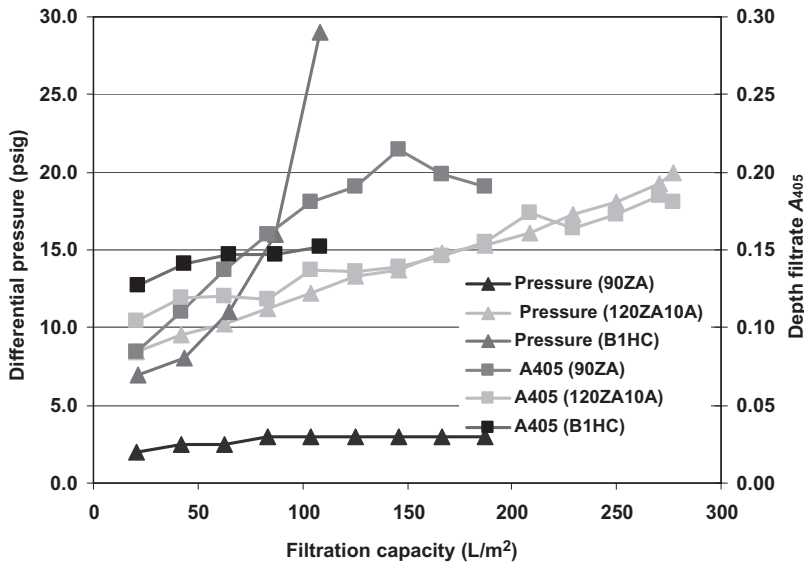
**FIGURE 3.8** Area under the absorbance trace at 410nm during Protein A elution vs. volumetric loading ( $\text{L}/\text{m}^2$ ) for one and two depth filters, respectively, following centrifugation.

These experiments are carried out at a constant flux rate with increasing volumetric loading. An example of the data from such a screening experiment is shown in Fig. 3.9, in which the lowest pressure drop and the highest clarification efficiency did not occur together for any given filter. In this example, the CUNO 120ZA10A can be regarded as a reasonable compromise between clarification efficiency and pressure drop.

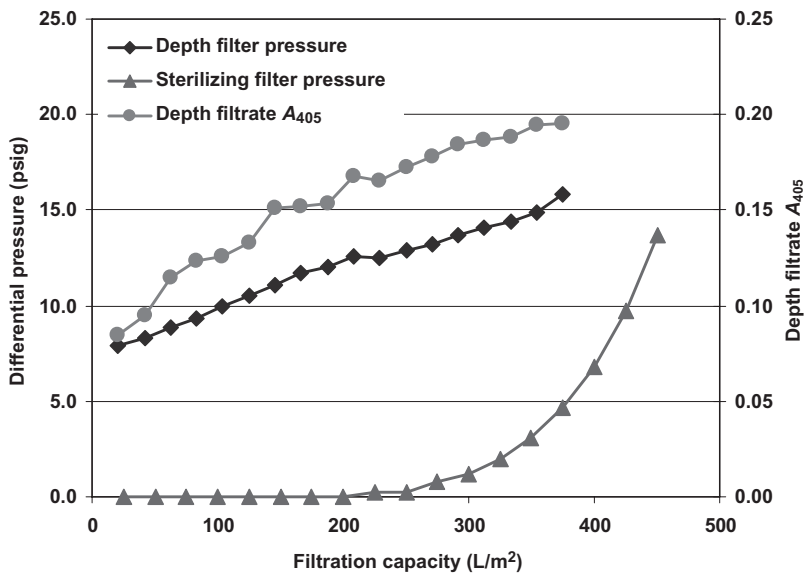
A more effective measure of clarification performance is the pressure drop across a dead-end microfilter after the depth filtration step. An example of such data is shown in Fig. 3.10, which plots the pressure drops across both the depth filter and the absolute filter. Effective clarification occurs even when loading the feedstream up to  $200 \text{ L}/\text{m}^2$ , with no operational issues in terms of excessive operating pressure. The efficacy of depth filtration and absolute filtration should be verified at the pilot scale before transferring to a manufacturing operation. Flow patterns in large-scale depth filters can be different from those of laboratory-scale devices due to the low-pressure conditions.

### 3.5 FLOCCULATION

Flocculants have been used for many years to improve the filtration characteristics of fermentation broths (13). Many flocculation agents have been used, ranging from simple electrolytes to synthetic polyelectrolytes. These agents can act in a number of ways to cause the clumping of smaller particulates into larger solids that can be filtered more effectively.



**FIGURE 3.9** Laboratory-scale screening of depth filters. A = CUNO 90ZA single layer: 90 (0.2–0.65  $\mu\text{m}$ ); B = CUNO 120ZA10A two layers: 90 (0.2–0.65  $\mu\text{m}$ ), 120 (0.1–0.45  $\mu\text{m}$ ); C = Millipore B1HC three layers: 50 DE (0.2–0.7  $\mu\text{m}$ ), 75 DE (0.1  $\mu\text{m}$ ), and RW01 (0.1- $\mu\text{m}$  membrane).



**FIGURE 3.10** Depth filtration over CUNO 120ZA10A followed by absolute filtration through a 0.2- $\mu\text{m}$ -rated in-line filter.

Filter aids have also been employed for the harvest of fermentation broths prior to filtration to remove biomass (35). These agents adsorb cells onto their surfaces, thus creating larger particles and decreasing the proportion of smaller particulates that can clog the filter pores. They also reduce the compressibility of the particles that are being removed, thus decreasing filter cake resistance. Two types of filter aids are particularly effective—diatomaceous earths and perlites. Both these materials include siliceous material that can adsorb cells and cell debris through a combination of charge-based and hydrophobic interactions (44). The one disadvantage of filter aids is the addition of large quantities of essentially earth-based materials to a clean fermentation broth. This is not so much of an issue with commodity products made from microbial cultures (e.g., penicillin), but it has restricted the use of such filter aids for mammalian cell systems. Therefore, filter aids immobilized on a support matrix (in the form of depth filters) are more commonly used to harvest mammalian cell cultures.

More recently, chitosan has been used as a flocculant for the clarification of mammalian cell culture broth, reflecting its status as a nontoxic, food-grade material (45). Centrifugation and depth filtration were carried out after the addition of chitosan, increasing the volumetric throughput of the depth filter six- to sevenfold.

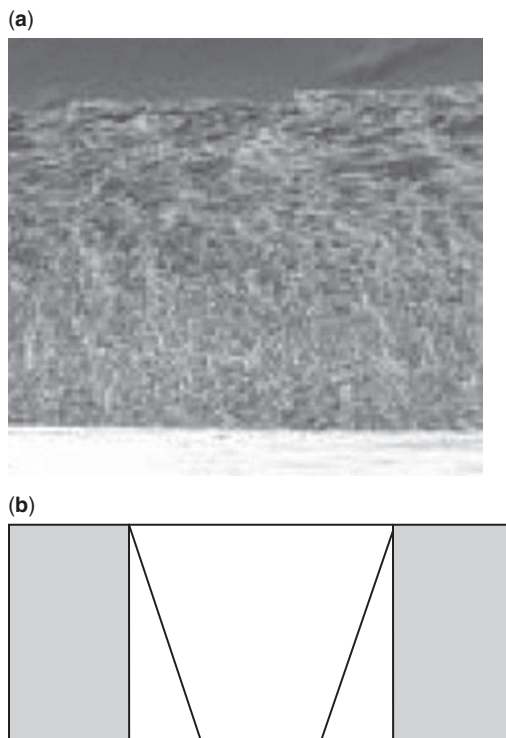
The combined use of a calcium chloride and potassium phosphate as a flocculant has also been reported, in this case with the dual purpose of clarification and the removal of soluble impurities (46). These two compounds when combined produce calcium phosphate, which is insoluble and can bind proteins through ionic and metal-chelate interactions.

Despite the successful studies described above, flocculation is not widely used for clarification during the manufacture of mAbs. However, interest in this process is likely to increase in the coming years since it is relatively inexpensive, and can reduce impurity levels as well as remove cells and debris, thus reducing the burden on the downstream chromatographic steps.

### 3.6 ABSOLUTE FILTRATION

Dead-end microfilters with pore sizes ranging from 0.2 to 1.0  $\mu\text{m}$  can be used for the removal of cells and cell debris from mammalian cell culture fluids. In practice, however, it is rare to see such filters used as the sole harvest technique beyond the laboratory scale since the surface area needed for efficient filtration at large scales can be prohibitive. For large-scale operations, dead-end filters are used as a terminal polishing step during clarification to ensure the absence of particulates in the load material for the capture chromatographic step.

Recent advances in the construction of dead-end filters have led to significant improvements in throughput and efficiency. Instead of a uniform pore size across the thickness of the filter, asymmetric membranes have been



**FIGURE 3.11** Schematic of an asymmetric membrane filter. **(a)** Scanning electron micrograph of an asymmetric membrane; **(b)** decrease of mean pore size from the open or lumen side into the membrane or the tight angle.

developed in which there is a graded decrease in pore size from the product side to the permeate side. This combines some elements of depth filter and absolute filter design, and significantly increases their capacity to handle biological streams (Fig. 3.11).

While comparing various brands of filters for clarification (Table 3.4), it is a good idea to determine their capacity at the laboratory scale. Based on the gradual pore-plugging model, a simple capacity evaluation can be carried out by measuring the volume filtered vs. the time at constant operating pressure (47). The equation for calculating  $V_{\max}$  (total volume that can be filtered per unit membrane area) is

$$\frac{t}{V} = \frac{t}{V_{\max}} + \frac{1}{Q_i} \quad (3.8)$$

A plot of  $t/V$  vs.  $t$  should yield a straight line with a slope of  $1/V_{\max}$  and an intercept of  $1/Q_i$  where  $t$  is the time;  $V$  is the cumulative volume filtered through the filter, and  $Q_i$  is the initial flux. This equation can be rearranged to

**TABLE 3.4 Absolute Filters Employed for Terminal Clarification and In-Process Filtration**

Vendor	Filter	Pore Size, μm	Description
Millipore	Durapore	0.22 and 0.45 + 0.22	Hydrophilic PVDF
Millipore	Express SHC	0.5 + 0.2	PES
Pall	Fluorodyne	0.1, 0.2	Hydrophilic PVDF
Pall	Supor EKV	0.2	PES
Pall	Ultipor N66	0.1–0.45	Nylon 6,6
Sartorius	Sartobran P	0.1, 0.2, and 0.45	Cellulose acetate with in-built prefiltration
Sartorius	Sartopore 2	0.1, 0.2, and 0.45	PES assymetric
CUNO	Bioassure	0.1, 0.2	Polyether sulfone (PES)
CUNO	SterAssure	0.1, 0.2	Nylon 66
Meissner	SteriLUX	0.1, 0.2, 0.4, 0.6	Hydrophilic PVDF
Meissner	EverLUX	0.2, 0.4, 0.6	Polyether sulfone (PES)

calculate the minimum filter surface area required to filter a given batch volume ( $V_B$ ) in a given processing time ( $t_B$ ):

$$A_{\min} = \frac{V_B}{V_{\max}} + \frac{V_B}{Q_i t_B} \tag{3.9}$$

This value can serve as a comparator for various membrane filters. Further experimentation at the pilot scale is often used to verify the accuracy of the filter sizing obtained by this methodology at the laboratory scale.

**3.7 EXPANDED-BED CHROMATOGRAPHY**

A chapter on mAb harvest and clarification would be incomplete without mentioning expanded-bed adsorption (EBA) chromatography. This technique has attracted significant interest over the years because separate harvest and clarification steps are not required (48–50). It involves the introduction of fermentation broth containing cells and debris into a column packed with the EBA resin, but in contrast to traditional methods, the broth flows upwards, causing fluidization of the resin so that the beads float, thus allowing the cells and debris to pass through and exit through the top adaptor while the product adsorbs onto the resin. After loading, the column is washed and allowed to settle. Product elution takes place in the downward flow direction. Some important considerations and limitations relevant to the development of EBA operations have been reviewed recently (51). Although of considerable interest, it is difficult to ensure uniform flow distribution from the bottom of the column as column diameters increase, and issues with frit and resin fouling have prevented the use of EBA for commercial-scale operations. If these

engineering issues are addressed in the future, EBA could perhaps reemerge as a favorable technique for harvest and capture in large-scale mammalian cell cultures.

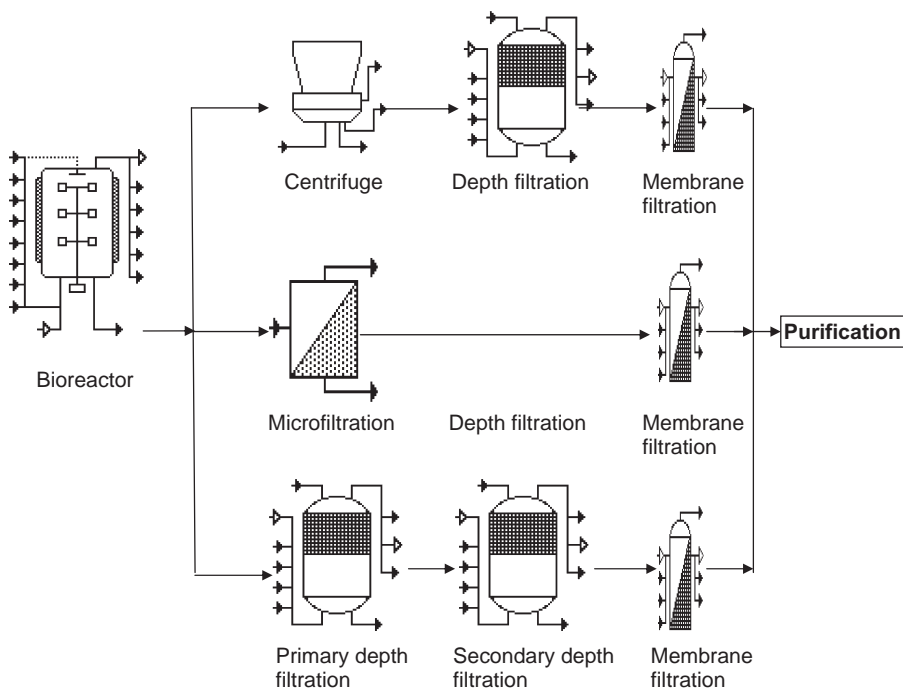
### **3.8 COMPARISON OF HARVEST AND CLARIFICATION UNIT OPERATIONS**

The appropriate combination of harvest and clarification unit operations for mammalian cell culture systems is dependent on production scale and on facility layout. Preferences have also evolved over time with increasing experience in scaling up certain unit operations. In the early 1990s, microfiltration-based harvest strategies were preferred because centrifugation was considered a significant capital investment and there was little experience in the control of shear stress on mammalian cells. Over time, better centrifuge designs and development efforts by both leading vendors (Westfalia and Alfa Laval) have helped to optimize operating conditions for biopharmaceutical cell culture broths, so centrifugation is now the favored harvest technique for cell culture facilities greater than 2000 L in scale. Disk-stack centrifuges are cleaner and easier to maintain in a sanitary state than in large-scale microfiltration housings. The capital investment in large-scale centrifuges is no longer seen as cost prohibitive in multiproduct facilities since the operating expenses with microfiltration membranes are significantly higher, and centrifuges can easily be changed over from one product to another (52). Bioburden control and cleaning validation are considered simpler for centrifuges compared with microfiltration systems since the latter have complex flow paths, which may incorporate dead zones that can harbor microbial growth.

The use of centrifuges does automatically imply the need for a secondary clarification step unlike microfiltration, which provides a clean filtrate stream. This niche is most commonly filled in mAb manufacturing processes by depth filtration. Large-scale depth filtration is now easy to scale up using process-scale housings and disposable filter modules. Recent work with flocculants and filter aids could increase the throughput of this step even further. However, it is rare to see depth filtration as the sole harvest technique at production scales greater than a few hundred liters. The terminal clarification step is almost always provided by in-line filtration through microfilters with an absolute pore size rating. These terminal filters ensure particle-free feedstock for the capture chromatographic step.

Harvest and clarification schemes for today's mAb production processes (Fig. 3.12) are the result of much development and evaluation carried out over the last 15 years. The last two decades have seen an explosion in the number of approved biopharmaceuticals as well as a significant increase in the number of mAbs produced at commercial scales (several hundred kilograms per year or more). This contrasts with the previous generation of biopharmaceuticals, where the production scales were much smaller due to the lower





**FIGURE 3.12** Common harvest and clarification schemes for large-scale mammalian cell culture harvest.

demand. This has driven increases in the scale of mammalian cell cultures, with bioreactors as large as 25000L now being pressed into service. The payoff for this ongoing work is that harvest techniques for mammalian cell culture systems now routinely operate with yields in excess of 98% with minimal cell disruption. Higher mAb titers and the larger number of mAb-based drugs on the market mean that these unit operations will be operated in similar fashion for some time to come. The challenge has now moved further downstream to improve the throughput of capture (see Chapters 4–6) and polishing (see Chapters 7 and 8). It also implies that the current cell culture scales are likely to stay with us over the next decade. Instead of radical changes to the way harvesting and clarification are carried out, improvements are likely to manifest as gains in efficiency and throughput, and improved understanding of existing unit operations.

### 3.9 ACKNOWLEDGMENTS

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# 4

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## PROTEIN A-BASED AFFINITY CHROMATOGRAPHY

SURESH VUNNUM, GANESH VEDANTHAM, AND BRIAN HUBBARD

### 4.1 INTRODUCTION

A variety of preparative chromatography modes have been used for the process-scale purification of monoclonal antibodies (mAbs). Most schemes have employed Protein A affinity chromatography as the capture step because of its specificity for the fragment-crystallizable (Fc) region of antibodies, its physicochemical stability, and the ease and simplicity of process development. The large purification factor obtained from this process step helps to simplify the entire downstream purification process. In general, only trace contaminants [high-molecular-weight (HMW) aggregates, residual host cell protein (HCP), and leached Protein A] remain to be removed after this unit operation, and such polishing can usually be achieved in one or two subsequent chromatographic steps (1, 2) (see Chapter 7).

As of early 2008, 21 mAbs have received approval by the Food and Drug Administration, while the global antibody pipeline comprises more than 350 antibodies in development. Antibody therapy typically involves frequent high doses, which, when combined with the large patient populations for some of the indications, can lead to a demand of several hundred kilograms or more of the product per year. Hence, cost-effective mAb manufacturing is gaining importance and is critical for the industry's success (3). Recent advances in cell line selection, growth and production media, feeding strategies, process control, and process design have led to expression levels as high as 5.0 g/L in a 12-d fed-batch process. Consequently, process bottlenecks have moved

downstream and the purification costs are now outbalancing cell culture costs (4). The Protein A resin alone constitutes approximately a quarter of the total cost of consumables in a downstream process for mAb production (5). Protein A affinity chromatography has to meet the demands of high dynamic binding capacity (DBC) and high throughput to keep pace with increasing bioreactor volumes and cell culture titers, in order to avoid becoming a process bottleneck. Thus, optimal usage of the Protein A affinity resin is critical to reduce the costs of mAb production.

In this chapter, we summarize the properties of the Protein A ligand, review commercially available Protein A resins, discuss the development and validation of the Protein A capture step, and finally address process-scale challenges and issues associated with this step.

## **4.2 PROPERTIES OF PROTEIN A AND COMMERCIALY AVAILABLE PROTEIN A RESINS**

### **4.2.1 Protein A Structure**

Protein A is a polypeptide anchored in the cell wall of *Staphylococcus aureus*. The architecture of the full-length Protein A molecule is characterized by a C-terminus that begins with a cell wall/membrane-associated region, proceeding into a linear series of five homologous antibody-binding domains. These domains are designated as E, D, A, B, C (in order from the N-terminus) and share 65%–90% sequence identity (6). The molecular weight of an intact molecule is 54 kDa, while that of a derivative with the cell wall domain deleted is ~42 kDa. The molecular weight of each of the antibody-binding domains is 6.6 kDa. Each domain of Protein A consists of an antiparallel three-helix bundle motif with two interhelical loops and has approximately the same antibody-binding ability.

### **4.2.2 Protein A–Immunoglobulin G (IgG) Interaction**

IgG binds to the individual domains of Protein A via its Fc region at the junction between the CH2 and CH3 domains (7). All the contacts seen in the X-ray structure of the Protein A–IgG complex are derived from residues in the first two helices, but helix 3 is critical for the stability of the Protein A–IgG interaction, which primarily consists of hydrophobic interactions along with some hydrogen bonding and two salt bridges (8). Studies reveal a highly conserved histidyl residue in the center of the Protein A-binding site of IgG. This residue aligns face to face with a complementary and similarly conserved histidyl residue on Protein A. At alkaline pH, these residues are uncharged and there are no restrictions on interfacial contact. The hydrophobic character of the uncharged imidazolium rings contributes to net hydrophobicity at the interface, strengthening the association. At low pH, the histidyl residues are fully charged, hydrophilic, and mutually repellent.

Although the primary binding site between IgG and Protein A is in the Fc region, an alternative binding site has also been identified whereby the D and E domains of Protein A bind to the constant region of Fab from all antibody classes (9). It has been suggested that antibodies with the kappa light chain (LC) bind more strongly than those bearing lambda LC. The alternative binding site has also been shown to be on the heavy chain (HC) variable region (10). In particular, the specific consensus sequence LYLQMNSL on the framework region between CDR2 and CDR3 of the HC has been shown to exhibit this behavior (11). The human mAbs with this sequence are assigned to the VH3 family (12). The variable region interactions in VH3 antibodies can significantly influence their binding affinity and consequently their elution pH. It has been suggested that the variable region interactions may even be stronger than Fc-mediated interactions in some cases (13).

Variations in affinity for Protein A among IgGs of different species and subclasses have been mentioned in the literature (14). Human IgG, for example, is bound with high affinity with the exception of IgG3. In addition, human mAbs of subclasses IgG1 and IgG4 bind more strongly than IgG2. Antibodies belonging to the same subclass have Fc regions with >95% sequence identity and are expected to have similar binding affinities. Protein A binding is unaffected by variation in glycosylation, even complete deglycosylation (15).

#### **4.2.3 Stoichiometry of Protein A–IgG Binding**

Theoretically, IgG molecules can bind to each of the five domains of Protein A. However, a free solution binding stoichiometry of “2.0–3.3” has been reported in literature (16, 17), suggesting that not all five domains of Protein A are simultaneously available for binding. The binding stoichiometry is expected to be even lower with immobilized Protein A resin.

#### **4.2.4 Protein A Stability**

Protein A has a high conformational stability and is remarkably resistant to physicochemical stress. Protein A is stable over a wide pH range (2.0–11.0) and is able to refold after treatment with denaturing solutions such as urea and guanidium salts. The lack of cysteine residues allows cleaning with reducing agents. The flexible random coil sequences that link the domains provide a fair degree of steric mobility but are susceptible to proteolytic cleavage (18). The binding domains themselves are protease resistant. The stability of the Protein A ligand is, however, sensitive to alkaline conditions.

#### **4.2.5 Commercial Protein A Resins**

Protein A sorbents are available from several commercial suppliers and vary with respect to the source of the Protein A ligand (natural wild type vs.



recombinant), immobilization chemistries, and bead characteristics. The two leading manufacturers of industrial Protein A chromatographic media are GE Healthcare and Millipore. Recombinant Protein A is expressed in *Escherichia coli* and is mostly derived from secreted extracellular variants that lack the cell wall/membrane-associated region. Various constructs incorporate different features to support directional coupling of the ligand to the solid-phase supports. Differences in matrix composition, bead size, and pore size can give rise to differences in resin compressibility, chemical resistance, permeability, available surface area, and mass transfer properties, which can all have an important effect on the performance of the Protein A step (19).

The rProtein A Sepharose FF resin uses recombinant Protein A that is coupled to the CNBr-activated Sepharose FF resin via the C-terminal cysteine through a single thioether linkage. Thioether coupling allows the ligand to extend farther into the mobile phase space than would be possible for a laterally immobilized ligand, and this improves antibody binding (20). In the case of rmpProtein A Sepharose FF resin, the recombinant Protein A is coupled to Sepharose FF resin through a multipoint attachment, which significantly reduces the amount of leaching compared to rProtein A Sepharose FF resin.

Sepharose materials have recently been improved further to generate a highly porous material that is also mechanically stable. MabSelect uses the same recombinant Protein A as rProtein A Sepharose FF, but it is attached to a highly cross-linked agarose matrix (21). MabSelect Xtra is based on highly cross-linked agarose and has a higher ligand density and a wider pore size than MabSelect, thus conferring a higher binding capacity (22).

A new resin, MabSelect SuRe, has recently been developed to withstand stronger alkaline conditions allowing the repeated use of 0.1–0.5 M NaOH for cleaning and sanitization (23). Using protein engineering techniques, a number of asparagine residues were replaced in the Z domain [a functional analogue and energy-minimized version of the B domain (24)] of Protein A, and a new ligand was created as a tetramer of four identical modified Z domains. The absence of D and E domains in the new resin also help eliminate variable region interactions and reduce binding heterogeneity between antibodies (25). However, the base matrices and ligand densities for the MabSelect and MabSelect SuRe are identical. It has been reported that the steric factor for IgG binding on Protein A can be slightly higher on MabSelect SuRe where the binding is through the Fc region, as compared with that on MabSelect, where the binding is through both the Fc and Fab regions (17). This may lead to subtle differences in the binding capacities on these resins.

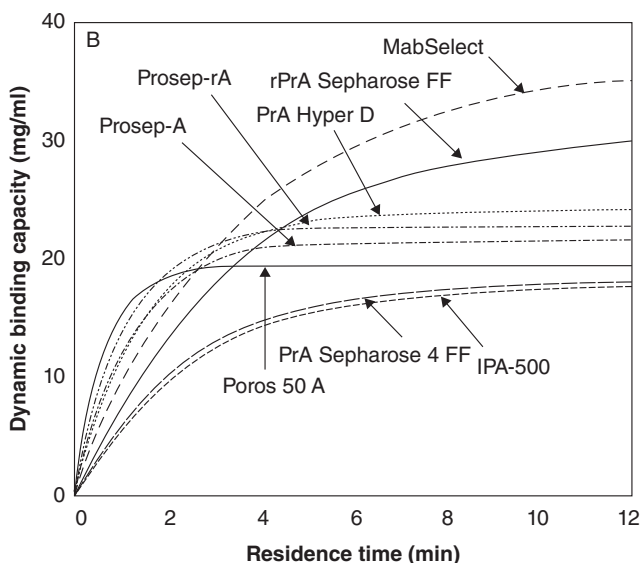
Protein A sorbents based on porous glass (ProSep A), coated porous polystyrene materials (POROS), and other types are also available. These materials are rigid and can be operated at high flow velocities. ProSep rA is based on controlled pore glass (CPG) with interconnecting pores of uniform size (100 nm). ProSep-vA Ultra has a pore size of 70 nm and has been designed to have a higher binding capacity due to the smaller pore and higher surface area compared with ProSep rA (19).

### 4.2.6 Static Capacity

The static capacity of IgGs on the various Protein A resins has been reported as follows: MabSelect Xtra ~ ProSep A Ultra High Cap > MabSelect ~ rSeph arose FF > ProSep A High Cap (26). This trend is in agreement with differences in stationary-phase morphology among the various resins. ProSepA Ultra High Cap has a higher static binding capacity than ProSepA high cap due to its larger surface area for binding. The ligand density (number of ligands per unit surface area) is identical for each resin (19). In another study, ProSep-vA Ultra has been shown to have a higher capacity than MabSelect SuRe (17). Maximum binding capacities at equilibrium for agarose-based media fall within the range 55–67 mg/mL (26).

### 4.2.7 DBC

The DBC of a human IgG is shown as a function of flow rate for a variety of commercial resins in Fig. 4.1. At residence times of more than 3–5 min, MabSelect and rProtein A Sepharose FF exhibit superior capacities over all other media. This is due to the high equilibrium binding capacity and low dissociation constants, and because of the larger particle diameters, which require longer residence times for mass transfer (26). ProSepA has a larger pore diameter and reduced surface area and consequently has better mass transfer properties compared with agarose-based media. The values of the effective diffusion coefficients for the ProSepA High Cap resin are almost an order of



**FIGURE 4.1** IgG DBC as a function of column residence time for commercial Protein A resins (26).

magnitude higher than those on agarose-based MabSelect media. However, the DBCs at breakthrough are lower on ProSep A compared with the agarose-based MabSelect media (17). ProSepA Ultra has a higher DBC than ProSepA because of the larger surface area available for binding. However, protein uptake has been found to be slower with ProSepA Ultra due to the smaller pore size (19).

The sensitivity of the DBC to variations in the load concentration can be quantified by the first derivative of the DBC with respect to the load concentration. The range of this derivative for all Protein A resins was 0–8, i.e., the maximum change in DBC for a load concentration variation of  $\pm 0.25$  mg/mL was  $< 2$  mg/mL, suggesting that the current generation of commercially available resins has a relatively high robustness against variations in load concentration (27).

#### 4.2.8 Leaching

One of the drawbacks of Protein A chromatography is that the Protein A ligand often coelutes with the antibody. Chromatography runs performed with harvested cell culture fluid (HCCF) often leach fragments of Protein A, as well as intact Protein A, into the column eluate. The leached Protein A fragments range in molecular weight from 6 to 40 kDa (18) and arise through proteolytic cleavage of the interdomain sequences by proteases in the HCCF.

Leached Protein A levels are typically higher for ProSep-vA Ultra than for MabSelect or MabSuRe. The leachate values are 3–5 ppm in the case MabSuRe, 3–35 ppm for MabSelect Xtra, and about 40 ppm for ProSep A Ultra (26). It has been suggested that the immobilization chemistry and/or the higher ligand density of MabSelect Xtra and ProSep-vA Ultra might be responsible for this finding. Another explanation for the less-profound leaching of MabSelect SuRe is the engineered ligand used for this matrix. It is worth noting that lateral attachment reduces access to interdomain cleavage sites, and multi-point linkages limit the release of proteolytic fragments. The multipoint-bound Protein A media maintain binding capacity over their lifetimes better than media with the ligand oriented for better antibody accessibility. Protein A fragment leaching can be inhibited to some extent by the addition of ethylenediaminetetraacetic acid (EDTA) to HCCF (the addition of EDTA inhibits metalloproteases, but other proteases may also be involved).

Proteolysis is not likely to be responsible for the leaching of intact Protein A. It may instead be due to free Protein A binding nonspecifically and non-covalently to IgG on the resin (followed by coelution with the antibody), to the dissolution of the base matrix during pH cycling (CPG in the ProSep A group of products) or due to physical or chemical breakage of the glycosidic linkages in the agarose base matrix (in the MabSelect group of products) (28). However, matrix degradation does not appear to be a major leaching pathway for agarose or other polymer-based media. It is a serious limitation for silica or CPG media, both of which dissolve at alkaline pH (29).

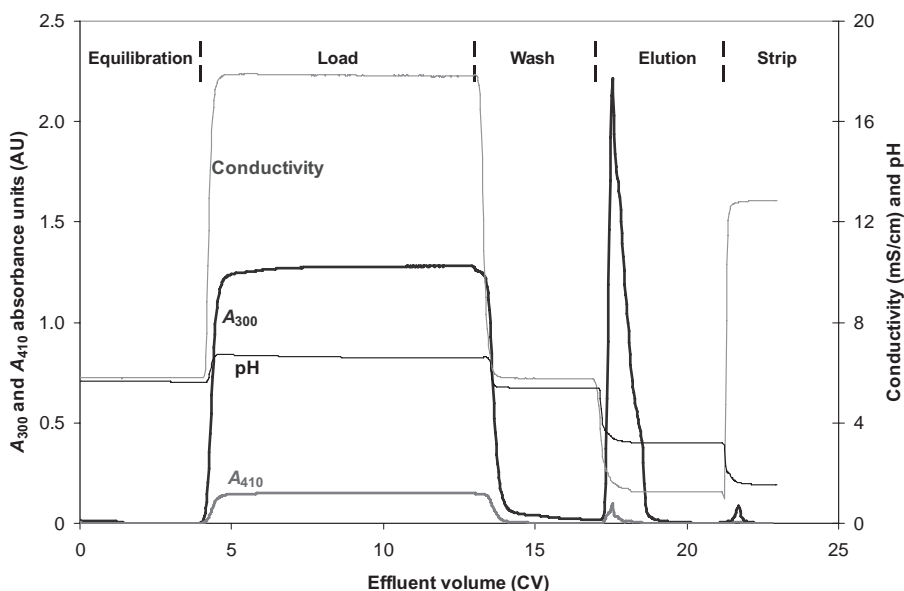
### 4.2.9 Production Rates

Volumetric production rate is defined as the amount of product purified per hour per liter of resin. ProSep A Ultra and ProSep A High Capacity resins have a higher maximum productivity than other compressible resins (19, 27, 30, 31). The operational window is larger for incompressible resins than it is for the compressible resins. Consequently, cycle time is much shorter for porous glass resins compared with cross-linked agarose resins. Although compressible resins have equal or higher DBCs compared to the evaluated incompressible resins, their productivity is lower because of the significant role that flow rate plays in the productivity calculations. The productivity of agarose-based media is higher at low flow velocities because the DBCs are higher, whereas the non-agarose-based media are more productive at high flow velocities (17, 26, 27, 32).

## 4.3 PROTEIN A CHROMATOGRAPHY STEP DEVELOPMENT

The objectives of the Protein A chromatography step in a mAb manufacturing process are product capture from HCCF and the removal of HCP, DNA, potential adventitious viral contaminants and process-related impurities. The Protein A step also serves as a key volume reduction step in the process, and is expected to provide 5- to 10-fold concentration of the product.

A typical Protein A chromatogram is shown in Fig. 4.2. The HCCF is loaded directly onto the column (at a neutral pH) and the product is eluted from the



**FIGURE 4.2** A typical Protein A chromatogram.

column at low pH. A wash step is introduced between column loading and elution to remove HCP and other contaminants. Finally, the column is stripped and regenerated for further use. The details of the key segments of Protein A column operation are discussed below.

#### 4.3.1 Loading/Binding

Most of the mAbs currently being used or investigated for therapeutic applications are human or humanized molecules belonging to IgG classes 1, 2, or 4 (33), all of which bind strongly to Protein A. The direct capture of mAbs from HCCF is therefore common practice in the biopharmaceutical industry, and presents no significant technical issues due to the high titers and efficient resins currently available on the market. An ultrafiltration/diafiltration (UF/DF) step can be included prior to Protein A chromatography if the titers are low and the DBCs are unacceptable. Alternatively, salt can be added to encourage mAb binding to Protein A resins (34). If need be, the pH of the column feed can be adjusted to reduce deamidation, and EDTA can be added to reduce proteolytic degradation, which leads to ligand loss.

Typical mAb purification processes run several cycles on a relatively small Protein A column to purify a single batch. This reduces capital costs if the column is compromised during operation, and also brings the column diameter within a practical range. However, cycling increases the total purification time, and hence processing time is an important consideration during Protein A step development (31).

The loading step accounts for a significant proportion of the overall processing time and is the rate-limiting stage of Protein A chromatography. The DBC of the product mAb is determined as a function of flow rate and residence time, and a loading flow rate that maximizes the overall process productivity is chosen. It must be noted that the differences in DBC as a function of flow rate/residence time (Fig. 4.1), as well as differences in the pressure–flow characteristics between the various Protein A media, can result in wide variations in process productivity (30, 31). During resin screening, one should therefore compare the performance of Protein A resins using both volumetric (amount of protein purified per unit time per unit column volume) and/or actual production rates at different residence times. The residence time expressions in these calculations can also be modified to accommodate the use of dual (or multiple) flow rates during the loading step (35). Irrespective of which loading method is used, the resin comparison can be extended further by including a consideration of pressure drop at higher velocities, resin costs, and the impact of column cycling (36). The final resin selection is based on the overall goals of the process in development and is typically a compromise between throughput, cost, impurity removal, Protein A leaching, flow characteristics, cleaning in place (CIP), and resin lifetime considerations (discussed below).

It is worth noting here that as achievable titers in cell culture processes increase, the choice of resin is based more on the DBC and less on how fast the column can be loaded. In this scenario, the Protein A step should be pressed to maximum loading capacity, which can be provided by relatively longer residence times, taller columns, and hopefully the development of new resins with greater capacities.

### 4.3.2 Wash Development

Although the support matrix should minimize nonspecific binding, this is rarely achieved in practice as derivatization of the support invariably introduces reactive groups. Indeed some base matrices, such as those used for ProSep-vA, can exhibit a significant degree of nonspecific binding. The immobilized Protein A, as well as the bound antibody with charged groups contributed by the side chains of some of the constituent amino acids, will cause the adsorbent to act as an ion exchanger, and other compounds may bind to the adsorbent by electrostatic interactions. Alternatively, impurities may bind to exposed hydrophobic regions of the antibody. Washing with a dilute buffer solution may not be sufficient to remove nonspecifically bound impurities (e.g., DNA, HCP, media components), and more effective methods may have to be adopted.

Several strategies have proven effective, and in a systematic approach to buffer selection, the simplest option should be evaluated first. The first choice would be to evaluate the pH of the post-load wash, ensuring that it falls between that of the loading and elution buffers. For maximum removal of nonspecifically bound material, the pH of the intermediate wash should be as low as possible but not so low as to initiate premature elution of the mAb. While investigating the pH of the wash buffer, it may also be beneficial to investigate the choice of buffer salt (37).

Where an intermediate pH wash is impractical or insufficient on its own to reduce impurities to acceptable levels, then the second step is to evaluate the addition of a salt, amino acids, detergent, or solvent to the intermediate wash buffer. Once the buffer additive is proven effective, the concentration can be optimized. The typical concentrations of the additives used are 0.5–1.0 M for NaCl and Na<sub>2</sub>SO<sub>4</sub>; 0.5–2.0 M for CaCl<sub>2</sub>, MgCl<sub>2</sub>, and MgSO<sub>4</sub>; 0.002%–0.02% (v/v) for detergents such as Tween 20; 10%–20% for organic solvents; 10%–20% for polyethylene glycol and polypropylene glycol; and 0.5 M for amino acids such as arginine and glycine.

A combination of these excipients can be tested if the above approaches are insufficient alone to achieve the required purity levels. Recently, success has been achieved using buffer combinations consisting of salts and detergents, salts and solvents, salts and polymers, and high concentrations of Tris buffer (38).

### 4.3.3 Elution

Elution pH is typically set at the highest possible value while maintaining high product yield. This is done to minimize product exposure to low-pH environments since even small changes in elution pH can adversely affect product stability. In addition, molecules that elute at low pH tend to form aggregates that have the potential to compromise drug safety and to complicate the development of the purification steps that follow Protein A chromatography.

A variety of strategies have been used to address the issue of aggregation and precipitation during Protein A elution. Antibodies that elute at lower pH often benefit from the use of elution-enhancing additives. High concentrations of NaCl have been used in the elution buffer to increase the elution pH (34). Ethylene glycol increases the elution pH by weakening hydrophobic interactions between the ligand and the product (39), and is nondenaturing for some proteins at concentrations up to 30%. However, this increases the viscosity of the feedstream and may reduce the flow rate. Urea is an effective hydrogen donor/acceptor that can outcompete the hydrogen bonds that stabilize the interaction between Protein A and IgG, and could possibly be used at low concentrations to facilitate elution. Histidine and imidazole have also been reported to moderate the elution pH on Protein A columns by competing with histidine at the Protein A–IgG binding site (40).

Another approach is modification of the Protein A elution buffer to make the buffer conditions more conducive to product stability, and this is often the simplest solution. Low concentrations of NaCl/Na<sub>2</sub>SO<sub>4</sub> could be included to counteract the tendency of some antibodies to precipitate at low ionic strength. Stabilizers such as arginine have also been added to the Protein A elution buffer to reduce aggregation (41).

Low-temperature operation can in some cases reduce product aggregation on Protein A columns. Yet another strategy is to reduce the slope of the pH transition from wash to elution buffers. Even though Protein A is operated under step-gradient conditions, the mixing of the two buffers creates a pH transition that can be steep or gradual depending on the choice of wash and elution buffers and their strengths. The low-pH stability of the product, as well as the elution volume requirements, must be taken into account when deciding whether pH transition should be made steeper or shallower.

The elution pH of antibodies can vary quite substantially between pH 3.0 and 4.5, even for mAbs that belong to the IgG1 and IgG2 subclasses. A recent explanation for this has been provided through the demonstration of mAb interactions with Protein A through their variable regions (13). It has also been shown that such variable region interactions can be eliminated by using a genetically modified Protein A ligand composed only of B domains (MabSelect SuRe). The elution pH is less extreme when using this modified ligand and can help to reduce aggregation problems. The change in the ligand (MabSelect vs. MabSelect SuRe) only influences the binding of antibodies to the resin and does not alter the purification factor.



#### 4.3.4 Stripping

The post-elution strip phase typically uses a buffer solution that is at a lower pH than the elution buffer to remove any tightly bound product or product-related HMW species. A strip buffer in the pH range 2.5–3.0 is typically used for agarose-based media, while lower pH buffer solutions could be used on CPG-based media. The Protein A ligand itself is quite stable under these conditions.

#### 4.3.5 Regeneration and CIP

The ability of the Protein A resin to withstand a significant number of column reuse cycles is an important performance factor given the high cost of this media, and yet the problem of defining a CIP protocol that balances effective cleaning with long lifetime is particularly delicate for these resins since Protein A is generally labile to commonly used CIP agents. The cost of the cleaning agent itself is another important and yet frequently underestimated aspect of processing costs.

Protein A column regeneration is typically carried out with high concentrations of chaotropes (e.g., 6M urea, 6M guanidine HCl) or low concentrations of NaOH (typically <100mM) (20, 26, 42, 43). Chaotropic solutions are costly and require special handling during disposal, while NaOH is inexpensive and easy to remove, and the removal is easy to monitor. Where possible, the vendors have therefore recommended regeneration protocols that use low concentrations of NaOH.

Native or recombinant Protein A is stable in slightly alkaline conditions; thus, low concentrations of NaOH can be used to clean Sepharose-based resins. Brorson and colleagues (42) have investigated the use of 100 and 500mM NaOH on Protein A Sepharose FF and found that after 50 cycles with 500mM NaOH regeneration, the yield dropped to about 50%, while with 100mM NaOH, the yield was stable at around 100% for 200 cycles and dropped to 50% after 300 cycles. Hale and colleagues (44) observed a capacity loss of about 1% for every regeneration cycle with 500mM NaOH using the same matrix.

NaOH in ethanol is another reagent that is very effective for CIP, but it causes a much greater loss of binding capacity (~8% per cycle) and is not recommended for use on a routine basis (43). Regeneration procedures using NaOH/NaCl combinations consistently deliver high purity and recovery on MabSelect for 300 cycles (45). The addition of NaCl to the NaOH solution has a significant positive effect on the stability of rProtein A. The decrease in DBC on MabSelect resin was 0% after 100 cycles, 4% after 200 cycles, and 11% after 300 cycles using 50mM NaOH/1M NaCl. In contrast, when 50mM NaOH was tested as a CIP solution without NaCl, the decrease in DBC after 61 cycles was 16% (44). In many processes, a lifetime of fewer than 200 cycles is still economically viable. With proper adjustments to the antibody elution and



strip, CIP with 50mM NaOH/1 M NaCl or even higher concentrations should be a good starting point for agarose-based recombinant Protein A resins. The MabSelect SuRe Protein A resin with the engineered ligand is designed to be stable under alkaline conditions and can withstand NaOH concentrations of up to 0.5M for column regeneration. The treatment of Protein A resins with NaOH in the 50–250mM range does not affect the subsequent leakage of Protein A from the affinity matrix.

The silica backbone of ProSep-vA Ultra is very unstable in alkaline conditions, and guanidine HCl is the preferred agent to regenerate these resins. Urea and guanidine HCl have also been used at very high concentrations when CIP using NaOH concentrations compatible with resin stability produces unacceptable levels of batch-to-batch carryover. Typically, 6M guanidine HCl or urea is used once every five cycles, or once per batch for a Protein A column cycled multiple times per batch due to cost and disposal issues. The extent of protein carryover is significantly reduced when these chaotropic agents are used, and the DBC of the resin is not affected (Amgen internal data). In cases where either urea or guanidine HCl alone has not been effective, the inclusion of low concentrations of a reducing agent such as dithiothreitol (DTT) has improved the results. The Protein A ligand does not contain any disulfide bonds, and is not impaired by reducing agents in the cleaning solutions.

#### **4.4 ADDITIONAL CONSIDERATIONS DURING DEVELOPMENT AND SCALE-UP**

Important additional considerations during the Protein A step include reducing HMW aggregate levels and eliminating turbidity from the Protein A peak pool, which occurs in most industrial mAb processes. As discussed in the previous section, one of the limitations of Protein A chromatography is the need to carry out product elution at low pH. Exposure to acidic conditions frequently results in the formation of soluble HMW species and/or insoluble precipitates during product elution. HMW aggregates can reduce the product yield and places an additional burden on the polishing steps to achieve clearance at the expense of overall process yield. Insoluble aggregates may form from the product and/or from impurities such as HCP. The insoluble particulates diffract light and cause the peak pool to appear turbid. The formation of insoluble aggregates can also increase the cost due to the filtration area needed to clarify the product stream.

Depending on the specific properties of the molecule involved, either the soluble or insoluble aggregates may dominate. In any case, the aggregates can reduce column lifetime if precipitation also occurs during elution. Additional aggregates and/or particulates may form during low-pH virus inactivation or during neutralization of the eluate pool. The inherently unpredictable nature of this precipitation phenomenon can lead to sporadic clogging during the sterile filtration step that follows neutralization of the Protein A eluate. A

depth filter is therefore often used to clarify the turbid neutralized Protein A eluate.

#### 4.4.1 Controlling HMW Formation

The primary means to avoid the formation of HMW aggregates during Protein A chromatography is to elute the bound product at as high a pH as possible without affecting product yield. This may be accomplished by adding modulators to the elution buffer (discussed above), or by using Protein A media with engineered ligands that facilitate the use of milder elution conditions. MabSuRe is a commercially available Protein A medium that interacts only with the Fc portion of the product (no Fab interaction), allowing the use of less-acidic elution buffers. Other engineered Protein A ligands vary the length of the loop region between helices 1 and 2 of the B domain, or introduce appropriate mutations on helices 1 and 2 so that Protein A–IgG interactions are disrupted under milder conditions (25). The elution pH is ~1.2 units higher for these engineered ligands as compared with native or recombinant Protein A.

Another operation that needs attention is the mixing of the elution pool. Proper mixing of the elution pool helps to dilute out the acidic end of the elution peak, reducing the duration of product contact under very low pH conditions, thus providing better control of the extent of HMW aggregate formation. Proper mixing is also critical during acidification of the Protein A peak pool for low-pH virus inactivation. Low-pH hold time studies indicate how sensitive the mAb is to HMW aggregate formation. Stabilizers may help to control aggregate levels, but the impact of stabilizers on the kinetics of virus inactivation must be evaluated. The concentration and type of titrant used for acidification, as well as the product pool concentration, could also influence HMW aggregate levels and must be studied carefully. Product pool dilution may sometimes be necessary prior to acidification.

#### 4.4.2 Removal of Soluble HMW Contaminants

The levels of product-related soluble HMW aggregates in HCCF vary between 0.5% and 25% for mAbs. The soluble HMW species also span a range of molecular weights, as shown by size exclusion and high-performance liquid chromatography (SEC-HPLC). The binding behavior of HMW species on Protein A is therefore quite complex. The diffusional resistance and extent of steric shielding of the immobilized ligand is expected to be higher for HMW species than for the corresponding monomer product because of the former's larger hydrodynamic radius, leading to a significantly lower DBC for the HMW species. On the other hand, due to the greater number of exposed binding sites, HMW species typically bind to the Protein A resin with greater avidity. For programs with high levels of HMW species, this could lead to an interesting scenario where the HMW species breaks through earlier than the

product during the loading phase, but once bound, the HMW species elutes later than the product.

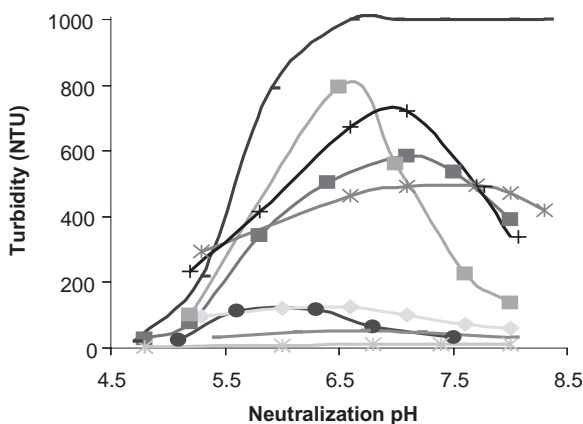
The optimization of the wash and elution phases of column operation can reduce the levels of HMW species in the product pool. The selectivity of the “product–HMW” separation can be improved with an appropriate choice of elution buffer (e.g., glycine, citrate, acetate), elution pH, salt type (e.g., NaCl, Na<sub>2</sub>SO<sub>4</sub>), and salt concentration. The elution conditions are chosen so that the product is eluted and the HMW species retained. The optimization of the wash phase is also critical as this determines the pH transition at the interface and hence the elution pool volumes.

The prepeak volumes are typically lower for programs with very high levels of HMW species (for the reasons discussed above) and consequently, the elution peak volumes may be larger. The large peak volumes and the inclusion of a greater portion of the wash in the final peak pool could have consequences in process integration and must be investigated carefully.

#### 4.4.3 Turbidity

Given the high load challenges on Protein A columns (30–45 g/L), the concentration of the product at the elution peak apex can be quite high, and the peak apex is often turbid. The  $A_{410}$  values at the peak apex can be as high as 5 OD units and the highly turbid elution pool can, in some instances, lead to clogging of the in-line filters.

The peak pool turbidities for several representative mAb processes are shown in Fig. 4.3 as a function of neutralization pH. The peak pool  $A_{410}$  values increase significantly upon neutralization. Following the low-pH inactivation step, the Protein A peak pool is adjusted to an appropriate pH and processed on the next unit operation in the downstream process. As can be seen in the



**FIGURE 4.3** Protein A peak pool turbidity as a function of pH for several mAbs.

figure, the extent of Protein A peak pool precipitation is quite variable from one mAb to another, and at times also between different batches of the same mAb. The correlation between peak pool turbidity and the  $V_{\max}$  values of in-line filters is neither predictable nor consistent.

As shown in Fig. 4.3, the impact of peak pool turbidity on the downstream steps depends on the operating pH of the second chromatography step in the downstream process. In some cases, the product yield can be near 100% even with the peak pool turbidity issues discussed here, thereby confounding the general notion that it is all induced by the product. In contrast to what one might expect for an affinity chromatography step, the HCP levels in the product pool can be as high as 50,000–70,000 ppm (46). Thus, insoluble particle formation during Protein A elution may not always be an undesirable phenomenon to be avoided at all costs.

Tobler and colleagues (47) systematically characterized the precipitate from the Protein A peak pool and concluded that a significant proportion of the precipitate was proteinaceous (primarily containing HCP and mAb). Two-dimensional gels of the peak pool precipitates confirmed that HCP levels were enriched in the precipitate phase relative to the peak pool. Lipids and DNA in cell culture media can arise from extensive cell lysis during cell culture or harvest operations and have sometimes been implicated in turbidity phenomena in downstream processing. However, the levels of DNA, lipid, and anti-foam in the precipitate samples were significantly lower than that of the proteinaceous material. Yigzaw and colleagues (46) addressed the possibility that cellular DNA could be responsible for enhanced turbidity during Protein A elution. They spiked HCCF with calf thymus DNA and processed it on a Protein A column. The addition of significant levels of DNA did not produce any increase in the turbidity of the Protein A elution pool. Treatment with DNase also had no impact. They concluded from this study that DNA is not the impurity responsible for the precipitation observed during Protein A elution.

It was concluded by both of these groups that HCP contaminants, and a small subpopulation of product that is improperly folded, might be responsible for Protein A peak pool precipitation. The problem may even be exaggerated if HCCF contains high levels of HMW species, or if the product is relatively unstable under the chosen elution conditions. This is to be expected since most mAb cell culture processes are long in duration, and are carried out at relatively higher temperatures, using high-cell-mass and low-viability fed-batch processes. It is possible that a small proportion of the product is denatured at 31–37°C in the bioreactor, and the HCP can associate with the exposed hydrophobic sites on the product. The HMW fractions in most mAb processes invariably have higher levels of HCP for this reason as well. In the context of Protein A step development, if the precipitating species is an HCP or a misfolded product, there may be the perfect opportunity to remove such contaminants by using depth filters downstream of the Protein A step to clarify the product stream and to clear proteinaceous impurities. The risk in this situation

is that particulate matter might clog the in-process sterile filters or might reduce the Protein A resin lifetime if the particles get entrained on the column during elution. On the other hand, if a measurable proportion of the product itself is lost through particulate formation, then product activity and process reproducibility concerns may arise. One way to distinguish between those two situations is by processing the Protein A eluate from one run on a second Protein A column. If the turbidity/precipitation does not recur in the second pass, then processing on Protein A itself is not deleterious to the product. In such a case, the bulk of the product species removed on the first pass through Protein A may be causing the problem. On the other hand, if the turbidity does recur on the second pass, it is likely that the product itself is unstable under the conditions encountered during Protein A chromatography (39). In such cases, milder elution conditions or the use of stabilizers should be considered, as discussed above.

At times, even if the product itself is stable under the elution conditions, the presence of HCP and/or misfolded mAb can lead to significant yield losses due to product coprecipitation. Appropriate selection of the harvest depth filter chemistry, flux, and volumetric loading has been shown to help reduce the level of turbidity and HCP in the Protein A column eluate. Depth filters are used post centrifugation in most clarification trains and are easy to implement on a large scale. The use of calcium phosphate flocculation was shown to decrease the Protein A peak precipitation for several antibodies (48). The turbidity of the Protein A peak was reduced significantly, and the inclusion of a flocculation step achieved a further 2 log reduction of HCP compared to a control run without flocculation. The use of Polymyxin-P at 0%–0.05% concentration, followed by centrifugation and depth filtration, also greatly improved Protein A elution and neutralized peak pool turbidity and filterability [49 (and Amgen, unpublished data)]. Similarly, the use of low-pH precipitation followed by centrifugation and depth filtration also improved the results (50). Thus, the incorporation of a wash step or the inclusion of adsorptive depth filters, low-pH precipitation, polyethylenimine, or calcium phosphate flocculation prior to the Protein A step may significantly improve Protein A process performance in the case where significant product loss occurs because of coprecipitation of the product.

#### **4.5 VIRUS REMOVAL/INACTIVATION**

The ICH Q5A guidance document requires the use of at least two dedicated orthogonal steps for viral reduction in addition to the clearance achieved by chromatography to ensure the safety of products produced by mammalian cell culture (51) (see Chapter 8). Given that the bound product during the Protein A step is eluted using a low-pH solution, the elution buffer can itself inactivate viruses, making this step both a removal and an inactivation step. However, since most mAbs are stable for a reasonable time under low pH conditions, a

dedicated low-pH incubation step is typically included in antibody purification processes to completely inactivate enveloped viruses, including the retrovirus models of the CHO cell type A nonviable retrovirus-like particles.

The removal and inactivation factors obtained through Protein A chromatography and low-pH incubation are analyzed individually because they involve different mechanisms and are considered to be orthogonal steps for virus clearance. A quantitative polymerase chain reaction (Q-PCR) assay is used to measure virus removal across the Protein A step, and when combined with infectivity assays, this can help to distinguish between removal and inactivation.

#### **4.5.1 Virus Removal**

The xenotropic murine leukemia virus (X-MuLV) log reduction values (LRVs) under standard Protein A conditions are typically in the range 1.0–3.5, while LRVs for minute mouse virus (MMV) are in the range 0.0–3.0. These values are lower than expected for an affinity chromatography step, and this may reflect nonspecific interactions with the resin backbone or binding of the virus to the bound product. The Protein A step can be optimized for virus removal with the incorporation of excipient washes similar to those that reduce peak pool turbidity or HCP levels. Modest improvements in X-MuLV removal values were noted with the incorporation of a high salt wash as compared to control run with an equilibration wash (Amgen internal data).

#### **4.5.2 Low-pH Inactivation**

The pH of the Protein A elution pool is typically adjusted to between 3.0 and 4.0 by adding an acid solution. The actual pH of the inactivation step is determined by the stability of the product, while the duration of the incubation is determined by the inactivation kinetics in the solution. Following acid inactivation, the solution is adjusted to an appropriate pH and is processed further.

Low-pH treatment has been shown to successfully inactivate many enveloped viruses in a variety of biotechnology products, including the retrovirus models of the CHO cell type A nonviable retrovirus-like particles (52). In one study, pH 3.5–4.0 was sufficient to inactivate X-MuLV at 18–26°C, and very little difference in the inactivation kinetics was observed between pH 3.7 and 4.1. At 2–8°C, however, pH 4.1 inactivation required up to 1 h compared to about 30 min at pH 3.7. Variability in X-MuLV inactivation kinetics was observed with different proteins. The inactivation time for one product was 1 h; for another, it was 120 min, and in yet another product, X-MuLV was not completely inactivated even after 120 min. Protein concentration also affected the inactivation kinetics. In buffer only, X-MuLV was inactivated in 120 min. The addition of protein prevented complete inactivation with the same pH, temperature, and exposure time. The ionic strength of the inactivating solution appeared to mitigate the effect of increasing protein concentration (53). In

another study, low pH was used to inactivate X-MuLV and pseudorabies virus (PRV) in several mAbs produced in either Sp2/0 or NS0 mouse cell lines. These data were used to support generic virus inactivation approaches (54). A different study of 13 products, mostly but not all mAbs, showed that incubation at pH 3.6–4.0 was sufficient to inactivate several viruses in 5–60 min (55).

#### **4.5.3 Bovine/Transmissible Spongiform Encephalopathy (BSE/TSE) Clearance**

Bovine-derived raw materials are used in the manufacturing process for many biopharmaceutical products. Such materials are sourced from countries considered to be BSE/TSE-free and are derived from low-BSE/TSE-risk tissues. However, health authorities require manufacturers of biopharmaceuticals to provide data supporting the safety of their products with respect to BSE/TSE risk. Protein A chromatography was shown to achieve a 2.2 LRV of BSE/TSE. The majority of the prion protein (>99%) was recovered prior to product elution in the flow-through and wash steps (56). Modern Protein A resins are now manufactured under conditions that are free from animal-derived components.

### **4.6 VALIDATION AND ROBUSTNESS**

#### **4.6.1 Validation**

Regulations demand that the reproducibility of the purification process with respect to the removal of specific contaminants is demonstrated during process validation studies, including consistency with reuse of the columns. Cycling studies are typically carried out on a small-scale column to establish that the performance of the Protein A step with respect to impurity removal, including virus clearance, is acceptable for the lifetime of the resin. Several recent studies with Protein A chromatography found that impurity removal, as well as viral clearance, was remarkably stable with extensive resin cycling (57). The Protein A resin is typically used for more than 200 cycles in commercial mAb manufacturing processes.

Given the complexity and cost associated with performing virus validation studies, Brorson and colleagues have recently proposed an alternative methodology where virus removal validation studies are performed on new media only, and chromatography performance attributes that decay simultaneously with, or prior to, virus removal are monitored during production, in lieu of performing virus removal validation studies with cycled Protein A media. This approach requires the identification of an appropriate performance attribute, but obviates the need for measuring virus LRVs in used media on a product-by-product basis (42). The performance attribute that best predicted the



column media lifespan was column capacity, as measured by mAb step yield and breakthrough, and not by impurity content. The step yield and product breakthrough values decay prior to retrovirus removal. Periodic evaluation of operating pressure and media packing, such as frontal analysis or height equivalent to the theoretical plate (HETP) analysis for used media, is warranted for the implementation of this approach. Note, however, that at the time of writing, the authors are not aware of any programs where this approach has been approved for Biologics Licensing Application (BLA) filings.

The ICH Q5A virus safety guidelines stipulate that adequate assurance must be provided that any virus potentially retained by the unit operation is adequately destroyed or removed prior to reuse (51). Such evidence for the Protein A step is provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

#### **4.6.2 Robustness**

The typical operating variables to consider for the evaluation of Protein A robustness include load challenge, flow rate/residence time, wash volume, wash buffer pH and concentration, elution pH, and feedstream variation (e.g., longer duration cell culture, low viability, higher titer). The performance parameters to look at include step yield, wash and strip peak area, prepeak volume, preneutralized peak pH, preneutralized peak turbidity, volume of titrant used, neutralized peak turbidity, and peak pool concentration. Operating conditions that lead to low prepeak volumes and high titrant requirements may, in some cases, affect process integration and downstream step performance, while high neutralized peak pool turbidity reduces the overall process yield. The product quality attributes to look for during robustness evaluation include the percentage of HMW and low-molecular-weight (LMW) product-related species, acidic and basic species, leached Protein A, HCP, DNA, and product activity. Runs with the worst product quality can be processed in the subsequent chromatography steps to evaluate process impact.

The impact of resin reuse on robustness must also be considered. If this is not feasible, the resin can be exposed to the strip/regeneration buffers for a duration that corresponds to the lifetime of the resin prior to robustness evaluation. Alternatively, the robustness runs can also be carried out as part of the resin reuse evaluation. The robustness runs can be performed in blocks and can be alternated with control runs operated at the set points (58).

### **4.7 CONCLUSIONS**

Cost-effective manufacturing is gaining importance with the growing number of mAb candidates currently in development. To this end, Protein A resin is often highlighted as the most expensive component as it contributes a quarter of the overall consumable costs in mAb downstream processes (5). Several



groups have therefore evaluated the feasibility of replacing Protein A chromatography in mAb downstream processes, and have shown that purification trains with three nonaffinity chromatographic steps can remove impurities to levels comparable to the traditional Protein A processes (59) (see Chapter 5). However, such purification schemes have not caught on in commercial downstream operations because the purification sequence must be designed on a case-by-case basis for each mAb and because there are challenges associated with fitting these processes into multiproduct facilities.

There has also been a considerable effort to identify low-cost mimetics that provide greater chemical stability and longer lifetime, and yet retain the simplicity and convenience associated with Protein A chromatography. The alternative ligands that have been identified have shown some selectivity, but the purification factors are significantly lower than those obtained with Protein A (60, 61). Thus, at this point, none of the small-molecule ligands can universally match the selectivity offered by Protein A for mAb purification.

The perception that the *up-front cost* of Protein A resin is a major expense in the commercial manufacture of mAbs is worth further comment. The resin cost is about a \$1 per gram, using assumptions that are consistent with the reported properties of commercially available Protein A media (DBC of 50 g/L and resin lifetime of about 200 cycles), and is really not a major driver for change (5). The cost of Protein A resin is significant for short-duration campaigns typically used for clinical programs. However, the advantages of process harmonization, and the use of template processes, often outweigh Protein A cost considerations in early development. An alternative approach for clinical programs may be to pack smaller Protein A columns and to use a simplified version of Simulated Moving Bed (SMB) chromatography (62) (see Chapter 11).

The *operating* expenses with Protein A, on the other hand, can be high depending on the cleaning solutions used. The conventional Protein A ligands cannot be exposed to alkaline conditions that are commonly employed to sanitize other column modes and often require high concentrations of chaotropes such as urea or guanidine hydrochloride for column regeneration and sanitization. This also creates disposal challenges and leads to high operating expenses. However, recent advances in ligand engineering have led to the development of base-stable Protein A media, thereby lowering the operating expenses associated with resin cleaning and reuse. The advantages of using a Protein A affinity capture step in mAb downstream processes therefore far outweigh the perceived concerns with this step. Protein A chromatography will continue to be the workhorse for mAb purification for the foreseeable future.

## 4.8 ACKNOWLEDGMENTS

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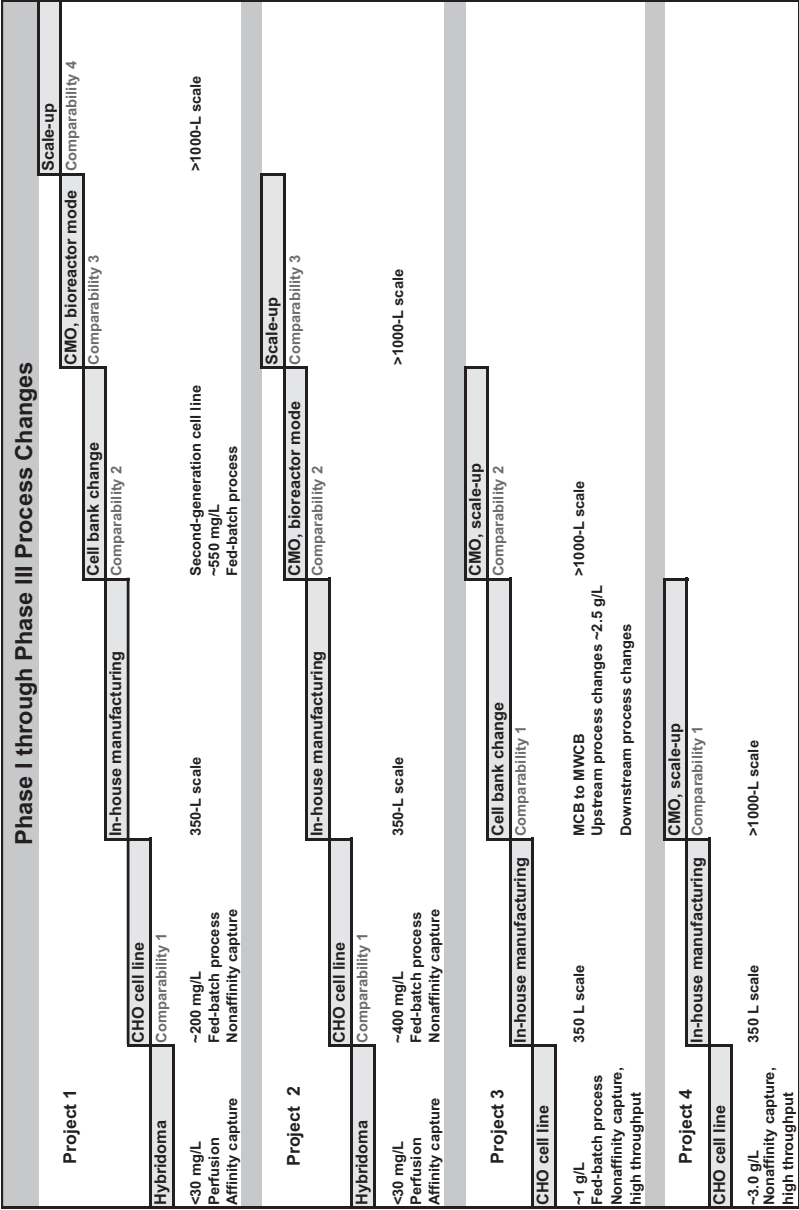
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## **PURIFICATION OF HUMAN MONOCLONAL ANTIBODIES: NON-PROTEIN A STRATEGIES**

ALAHARI ARUNAKUMARI AND JUE WANG

### **5.1 INTRODUCTION**

Monoclonal antibodies and their derivatives are major revenue generators in the global biotechnology market, with several antibodies already approved as therapeutics and a robust candidate pipeline comprising more than 200 antibodies at different clinical stages. The use of platform technologies has been very successful in developing processes for commercial antibody manufacture, especially Chinese hamster ovary (CHO) cells as the expression host and affinity purification as a key downstream process. Methods, tools, and technologies for large-scale antibody manufacture have been tailored to suit both current needs and future applications. The integration of interdisciplinary expertise in the specialized areas of molecular biology, cell line development and engineering, and cell culture optimization has significantly enhanced antibody yields (1). However, the benefits derived from such impressive upstream gains can only be realized in full when downstream productivity is similarly enhanced. The impact of high-titer processes integrated with highly efficient purification schemes has become evident not only in terms of production economics but also with respect to the overall strategy of process and product development. These high-yielding processes provide an opportunity to reduce both the time to market and the cost of goods for recombinant antibodies at the same time. Integrated process designs with comprehensive control strategies can also generate effective regulatory strategies. Consequently, overall drug development timelines and costs are reduced significantly (Fig. 5.1).

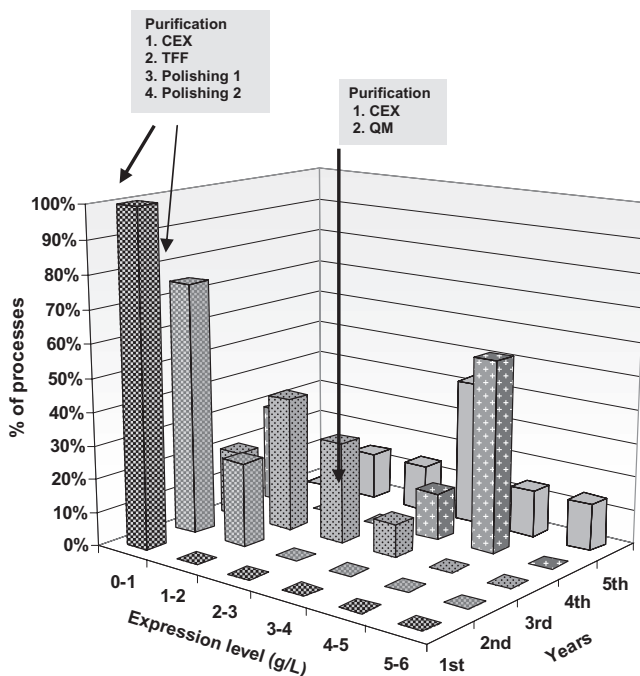


**FIGURE 5.1** Integrated CHO cell culture and nonaffinity platform technologies and their impact on product development stages. MCB: Master Cell Bank; MWCB: Manufacturing Working Cell Bank; CMO: Contract Manufacturing Organization.

## 5.2 INTEGRATED PROCESS DESIGNS FOR HUMAN MONOCLONAL ANTIBODY (HuMab) PRODUCTION

HuMabs are the most advanced antibody types generated by mouse technology (2) or phage display technology (3) that contain fully human protein sequences. They are increasingly favored as human therapeutics because they are unlikely to show immunogenicity. At the same time, HuMabs are steadily replacing older chimeric and humanized antibodies as second-generation candidates for already approved drugs. For example, Humira and Vectibix are approved HuMabs for the treatment of autoimmune disorders and cancer, respectively.

For HuMab production, a shift in the demand for productivity improvements from upstream to downstream processes has been recognized, and has been accommodated by simplifying purification processes to balance out the overall process productivity. Integrated strategies with more productive downstream processes were essential for the seamless transition between high-titer upstream production and high-efficiency purification in order to maximize the manufacturing efficiency (Fig. 5.2). Alternatives to affinity processes with improved process economics and higher throughput have been very successful (4).



**FIGURE 5.2** Progressive improvements in expression levels and integration with ion-exchange purification scheme. QM: Q membrane; CEX: cation exchange; TFF: tangential flow filtration.



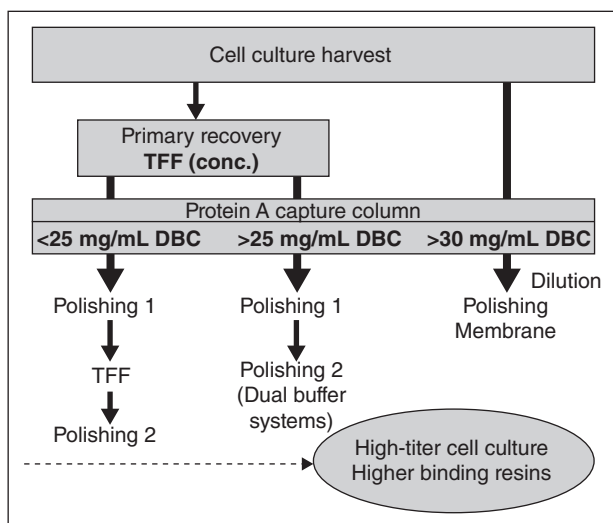
To date, purification schemes based on simple ion-exchange principles have proven to be robust and scalable (4) (see Chapter 10). Continuous modification of the purification strategy using a multipronged approach to handle expression levels increasing from 1 g/L to 6 g/L has led to a simplified process that, for certain HuMabs, comprises just two commonly used ion-exchange steps.

### 5.3 PURIFICATION PROCESS DESIGNS FOR HuMabs

#### 5.3.1 Protein A Purification Schemes

Protein A chromatography is currently the preferred method for capturing and purifying antibodies, antibody fragments, and IgG fusion proteins, providing flexibility for contaminant removal based on the affinity between Protein A and the Fc portion of the antibody molecule (5) (see Chapter 4). Despite the high affinity between ligand and target, the nonspecific binding of certain cell culture-related contaminants to Protein A requires further downstream process steps to achieve sufficient purity for therapeutic use. The number of polishing steps required for antibodies is generally defined not only by process-related contaminants but also by product-derived aggregates, modified product species, and degradation products.

Protein A processes have also been streamlined for HuMabs by using improved affinity resins and by removing several unit operations, in some cases resulting in a single capture step followed by a membrane chromatography step for polishing (Fig. 5.3). However, ligand leaching needs to be addressed,



**FIGURE 5.3** Development of simple two-step Protein A process schemes for HuMabs with only one column chromatography unit operation.

especially for high-titer processes with their increased contaminant loads, since there is only one polishing step after the affinity column. Recently, more stable Protein A resins that are less prone to leaching have provided better options for such short schemes. However, two-step purification processes with affinity capture followed by cation-exchange or mixed-mode chromatography are preferred where substantial amounts of leached Protein A need to be removed (6, 7).

Affinity chromatography has some well-known inherent limitations including high cost, the aforementioned problem with leaching, short operational lifetime, the potential for product denaturation, and the greater likelihood of aggregate formation and proteolysis. Bioaffinity ligands cost 7–15 times more than ion exchangers, and one affinity process step is nine times more expensive than a two-step ion-exchange process (8) even without considering the necessity for lot analysis to confirm that the product meets the specification for residual ligand levels. Nonaffinity alternatives offer better process economics with high-yielding upstream processes, and this justifies the investment required to develop efficient nonaffinity platform technologies for HuMabs.

### 5.3.2 Non-Protein A Purification Schemes

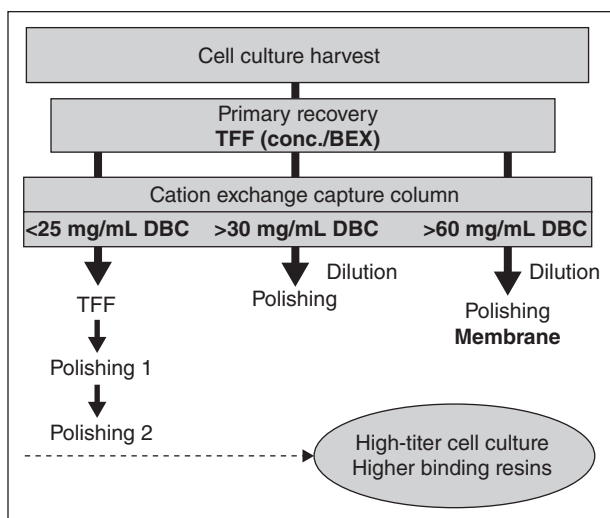
Economic non-Protein A alternatives that have been tested in the manufacture of antibodies include synthetic affinity ligands and the uniquely designed resin MEP HyperCel for selective binding to antibodies (9–11). Mimetic resins, composed of synthetic affinity ligands, have been employed as capture or polishing steps in the development of non-Protein A purification processes (12). The cost saving is the most significant advantage compared with Protein A resin, but other benefits include undetectable leaching, convenient cleaning, extended lifetime, and suitability for different HuMab types. The binding capacity of mimetic resins is a function of ligand density and spacer arm length, and it is also strongly influenced by the concentration of antibody in the load material. In most cases, the binding capacity is comparable to that of Protein A resin, in the range of 20–50 mg/mL resin, and the resin is capable of reducing aggregate levels. However, the frequently used mammalian cell culture shear protectant Pluronic F-68 interferes with the binding of HuMabs to mimetic resins due to the presence of competitive hydrophobic agents that weaken interactions with the hydrophobic moieties in the ligand itself. A cation-exchange step is therefore recommended to remove Pluronic F-68 prior to the mimetic resin, which is a major disadvantage in terms of replacing Protein A even though there may still be some cost advantage.

Unique resins have been introduced that can capture antibodies from high-ionic-strength feedstreams, and these are based on hydrophobic charge induction chromatography (HCIC). They reduce nonaffinity processes to simple two-step separation mechanisms (13). However, the resin binding capacity for HuMabs ranges from 20 to 30 mg/mL straight from conditioned media and requires multiple capture cycles. MEP HyperCel is an effective polishing step

for removing host cell contaminants and provides an excellent viral removal strategy for short purification schemes.

### 5.3.3 Host Cell Protein (HCP) Exclusion Approach for Ion-Exchange Purification Schemes

Ion-exchange chromatography is a common step in the purification of a wide variety of proteins expressed in different hosts, including *Escherichia coli*, yeast, and mammalian cell lines (14, 15) (see Chapters 6 and 7). Affinity capture-based processes for antibodies routinely employ cation- and anion-exchange polishing steps to reduce the level of antibody-derived contaminants (such as high-molecular-weight aggregates) as well as HCP, DNA, endotoxins, and Protein A leachate (16, 17). An entire purification process has been developed for the commercial-scale manufacture of Humira without the use of affinity chromatography (18). Three-column processes using cation-exchange, mixed-mode chromatography, or hydrophobic interaction chromatography have been evaluated as alternatives to affinity-based processes (19), and ion-exchange processes in particular offer economic viability by leveraging the lower resin cost, higher binding capacity, reduced cycle number, and greater longevity compared with the affinity capture step (2). The development of the nonaffinity platform combined with the continuous improvement of the CHO system through the exclusion of HCP is one of the major strategies contributing to the optimization of cation-exchange as a capture step. This makes it as efficient as affinity resins for the removal of contaminants, and eventually yields simple purification schemes (Fig. 5.4).



**FIGURE 5.4** Gradual removal of unit operations and replacement with single-use membrane polishing step. BEX: buffer exchange; DBC: dynamic binding capacity.

During process development, the degree of purification achieved in each chromatography step is important, and a process scheme evolves by integrating multiple process steps with orthogonal separation mechanisms in the most practical sequence, with interspersed feed conditioning steps (see Chapters 9 and 10). Orthogonal separation mechanisms are critical if the therapeutic product is to meet the set specifications for safety, purity, and potency (20). HCP is a major and complex contaminant that can provoke an immune response even if present at parts per million levels (21), and it therefore requires special attention at every stage of purification. To build a robust cation exchange-based process, the behavior of not only the antibody but also HCP under the relevant chromatographic conditions must be thoroughly understood. Continuous monitoring of HCP clearance levels after each chromatography step results in greater understanding and better control strategies during process development and scale-up.

HCP complexity and heterogeneity depend on the expression system or cell line and the cell culture conditions, and form part of defining the product profile for biologics. Platform technology development with a given cell line provides better insights into the nature of the contaminants and how they are cleared by a preferred purification process step. Therefore, analyzing the contaminant profiles for enrichment or clearance of HCP on a given resin can facilitate the development of specific methodologies to minimize HCP levels in the product. The binding, washing, and coelution patterns of contaminants along with a given antibody can provide useful information for the development and optimization of capture chromatography steps. This combined attention to the protein of interest and HCP is known as the HCP exclusion approach, and it allows cation-exchange chromatography to be optimized so it is as efficient for contaminant removal and product recovery as Protein A capture (Table 5.1). Optimization can, in certain cases, reduce HCP levels to below 100ppm and DNA levels to below 3pg/mg, which means only one more orthogonal purification step is required to meet therapeutic-grade product requirements (13). These simplified schemes not only reduce the capital costs of manufacturing but also reduce the validation costs.

**5.3.3.1 Primary Recovery.** Preconditioning of the load material is an essential step prior to capture by cation-exchange chromatography and is often carried out by diafiltration/tangential flow filtration (TFF) (22). In this unit operation, the antibody is not only concentrated into a lower volume but is also conditioned by buffer exchange to the appropriate pH and conductivity, allowing it to bind more efficiently to the capture resin. The intermediate (unprocessed bulk) from the primary recovery step between upstream and downstream processing is advantageous for HuMab manufacture because there is a gradual precipitation of DNA, cell culture components, and HCP as the pH of the conditioned medium is lowered by diafiltration, which serves as a partial purification step (Tables 5.1 and 5.2). The extent of this precipitation depends primarily on the pH and conductivity of the diafiltration buffer, and

**TABLE 5.1 Comparison of CHO HCP (CHOP) and DNA Removal in Protein A vs. Non-Protein A Purification Processes**

Process Step	CHO HCP, ng/mg		CHO DNA, pg/mg	
	Protein A	Non-Protein A	Protein A	Non-Protein A
CB <sup>a</sup>	$3.1 \times 10^5$	—	$5.8 \times 10^6$	—
UB <sup>b</sup>	—	$2.3 \times 10^5$	—	3998
Capture column	2135	888	500	3.3
Polishing column 1	12	35	28.9	0.3
Polishing column 2	8.5	Below detection	6	0.3

<sup>a</sup>CB was used as Protein A capture load.  
<sup>b</sup>UB, i.e., buffer-exchanged CB, used as non-Protein A capture load.  
CB = clarified bulk; UB = unprocessed bulk.

**TABLE 5.2 Normalizing Host Cell DNA by Primary Recovery Step for Cation-Exchange Capture Load**

Process Step	HuMab 1	HuMab 2	HuMab 3	HuMab 4	HuMab 5
CB <sup>a</sup>	$2.24 \times 10^7$	$3.80 \times 10^6$	$1.07 \times 10^7$	$1.11 \times 10^7$	$6.64 \times 10^6$
UB <sup>b</sup>	$4.12 \times 10^3$	$3.10 \times 10^4$	$6.72 \times 10^5$	$7.64 \times 10^3$	$1.17 \times 10^3$

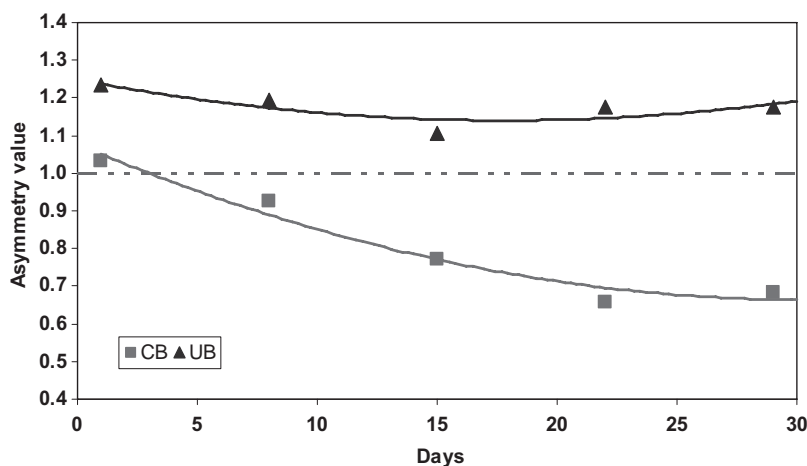
<sup>a</sup>CB from cell culture.  
<sup>b</sup>UB, i.e., buffer-exchanged CB.  
CB = clarified bulk; UB = unprocessed bulk.

the precipitate can be removed by depth filtration. Reduction of both DNA and HCP at this stage leverages the nonaffinity purification process by normalizing the ratio of host cell-derived contaminants to target HuMab levels as upstream productivity increases (Table 5.2). In order to reduce processing time, buffer-exchange steps can be performed using a diafiltration buffer with a lower pH (pH 5.8–6.0) and a lower conductivity than required at the end point (e.g., pH 6.2).

Several HuMabs are very stable in the unprocessed bulk from the concentration and diafiltration/TFF step compared to the initial cell culture harvest material (Table 5.3 and Fig. 5.5). This may reflect the removal of proteases from the cell culture supernatant during buffer exchange, especially for high-titer cell culture processes where cell densities tend to exceed  $1 \times 10^7$  cells/mL for several days in fed-batch cultures. Additionally, the low pH conditions commonly used for HuMab capture by cation-exchange chromatography help to prevent the secondary modification of proteins (e.g., deamidation and isomerization), which occur at high pH (23). Furthermore, this unprocessed bulk stage can be a convenient in-process hold step for high-yielding processes to facilitate better manufacturing schedules for multiple capture cycles.

**TABLE 5.3 Stability of Different HuMabs in Clarified Cell Culture vs. Buffer-Exchanged Cell Culture Supernatant Following Primary Recovery**

Product	Intermediate Storage, day	
	Clarified bulk	Unprocessed bulk
HuMab 1	1	28
HuMab 2	2	21
HuMab 3	14	28



**FIGURE 5.5** HuMab stability in clarified bulk (CB) and buffer-exchanged unprocessed bulk (UB) as assessed by HPLC-SEC monomer peak asymmetry value (value  $\geq 1$  indicates product integrity and stability).

**5.3.3.2 Optimization of Cation-Exchange Capture Chromatography.** Due to the presence of HCPs with a wide range of isoelectric points, the pH at which the load is conditioned will influence the level and type of HCP binding to the resin while the majority of the HCP will flow through according to the binding conditions. Turning cation exchange into a major workhorse for purification requires the optimization of loading and washing/clearance conditions, and the selective avoidance of HCP during product elution from the column, as described below.

**5.3.3.2.1 Influence of Binding Conditions.** One of the advantages of capture based on cation exchange is the choice of resins available and their extended binding capacity compared to affinity and selective resins. The binding capacity of some cation-exchange resins can be optimized by screening the type of buffer, e.g., phosphate, citrate, and acetate, and by testing the addition of NaCl to increase capture capacity by up to 50% (Table 5.4). As long as the post load

**TABLE 5.4    Influence of Buffer Species and Salt Concentration on the Binding Capacity of Cation-Exchange Resin at the Same pH and Conductivity**

Binding capacity (mg/mL resin) in different buffer species without NaCl	
Citrate, sodium phosphate buffer	26.9
Sodium phosphate buffer	39.0
Acetate, sodium phosphate buffer	57.3
Binding capacity (mg/mL resin) in the same buffer species with different NaCl	
Base buffer (no NaCl)	41.5
Base buffer + 25 mM NaCl	60.0
Base buffer + 45 mM NaCl	62.7

**TABLE 5.5    Influence of Different Load and Wash Conditions on CHOP Levels in Cation-Exchange Eluate**

Starting Material	Load pH	Wash Condition	Eluate CHOP, ng/mg
Buffer-exchanged	6.2	1	2,301
clarified bulk or	5.8	2	7,681
unprocessed bulk <sup>a</sup>	5.2	3	10,864
	4.8	4	18,464
	4.8	5	1,822
pH-titrated clarified bulk <sup>b</sup>	6.2	1	7,726

The CHOP levels in each of the materials (derived from the same clarified bulk) was <sup>a</sup>82,601 and <sup>b</sup>115,932 ng/mg.

wash conditions are set to optimize HCP clearance, the pH and conductivity can be reduced enough to maximize the binding capacity of the resin without HCP levels also increasing (Table 5.5). Buffer additives such as urea, ethylene glycol, glycine, and polyethylene glycol modulate the secondary interactions of ion-exchange resins and, in some cases, affect protein stability and solubility in a variety of ways (24). However, their influence on CHO HCP profiles requires further investigation.

*5.3.3.2.2 Clearance of Bound Contaminants.* Using selective solutions to eliminate contaminants from the resin can minimize HCP levels in the eluted product. Designing wash buffer compositions to reduce HCP and DNA levels plays an important role in developing cation-exchange capture efficiency, and the extent of this optimization dictates the number of polishing steps required after capture. Careful screening of wash buffers with different buffer species and a range of pH values and ionic strengths is essential for the efficient removal of bound contaminants. Out of all the process conditions, buffer pH is the critical parameter affecting the scalability and robustness of a nonaffinity manufacturing process. As shown in Table 5.5, wash buffer compositions can have a tremendous influence on HCP levels—e.g., binding at pH 4.8 and washing the column according to condition 4 yields 18,000-ng HCP per mg of protein, whereas the same binding pH combined with washing condition 5

reduces the level of HCP 10-fold. Washing condition 4 is obviously difficult to turn into a viable ion exchange-based purification scheme due to the heavy demand on downstream polishing steps, whereas washing condition 5 facilitates the development of a three- or two-step purification scheme. By monitoring HCP levels with different wash steps, effective process controls can be developed that are useful not only for the capture column but also for the entire nonaffinity purification scheme. The platform approach with a specific cell line is advantageous because optimization can be achieved for different HuMabs with slight modifications based on background information about HCP behavior.

**5.3.3.2.3 Product and Contaminant Coelution Profiles.** Residual HCP levels in the product can also be reduced by optimizing the elution buffer composition with respect to buffer strength and the NaCl concentration. The less salt present, the lower the levels of HCP (Table 5.6). Using the same binding pH conditions but eluting at a lower pH can also reduce the HCP levels, since most HCPs are then left on the column to be stripped (Table 5.7). In addition, analysis of the product elution peak by fractionating the eluate can help to identify contaminant trends in the profiles either at the beginning or trailing at the end of the peak. This helps to reduce the number of chromatography steps required for polishing. There seems to be an inverse correlation between product concentration and contaminant levels, especially for HCP (Fig. 5.6), which helps to define the elution conditions required for optimal product recovery and minimal HCP levels. In a three-column purification scheme, HCP levels can be reduced to approximately 2000ng/mg after cation exchange,

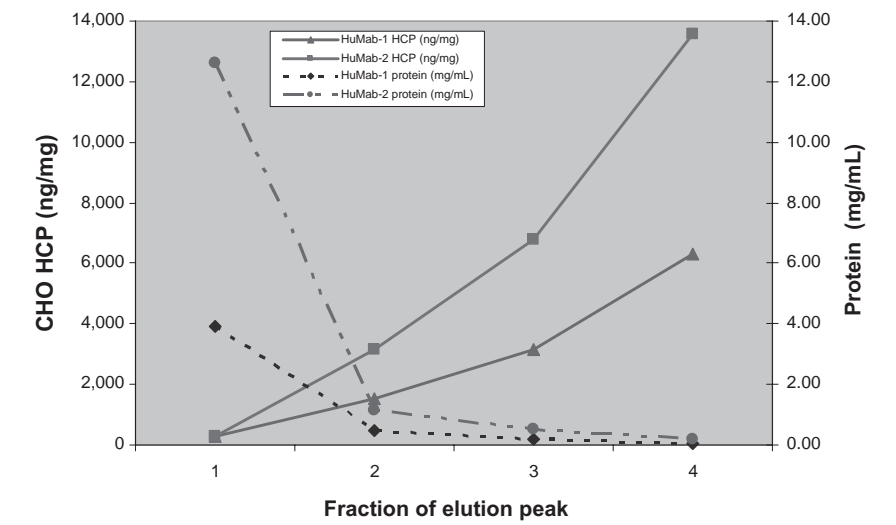
**TABLE 5.6 Effect of Different Elution Buffer Species on CHOP Removal by Cation-Exchange Capture**

Process Stage	CHOP (ng/mg) Using Buffer Composition 1		CHOP (ng/mg) Using Buffer Composition 2	
	Run 1	Run 2	Run 1	Run 2
Cation-exchange load	109,263	119,426	90,204	94,311
Cation-exchange elution	2,202	3,058	946	1,067

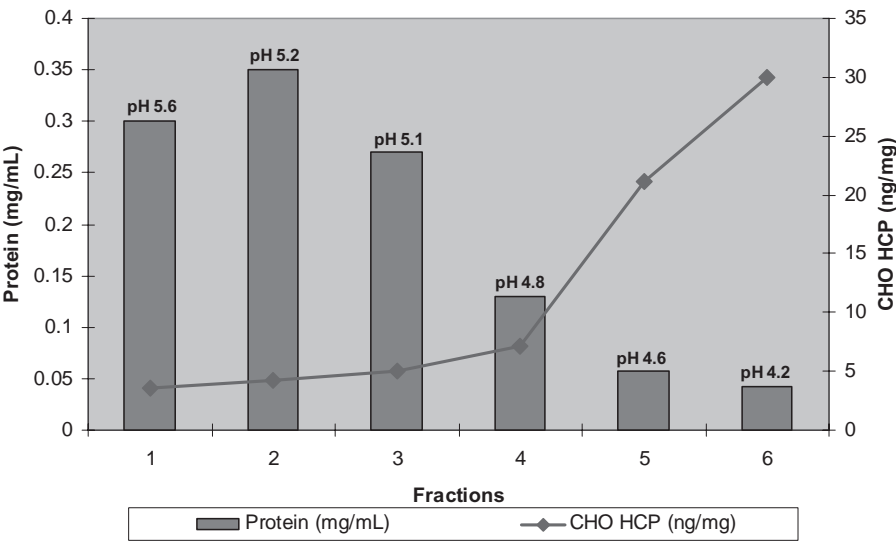
**TABLE 5.7 Coelution of Bound Contaminants by Lowering Elution pH Compared to Load pH**

	Condition 1	Condition 2	Condition 3
Load/wash 1	pH 7.0	pH 7.0	pH 7.0
Wash 2	None	pH 6.2	pH 4.5
Elution	pH 7.0	pH 6.2	pH 4.5
CHOP, ng/mg	5534	1920	908



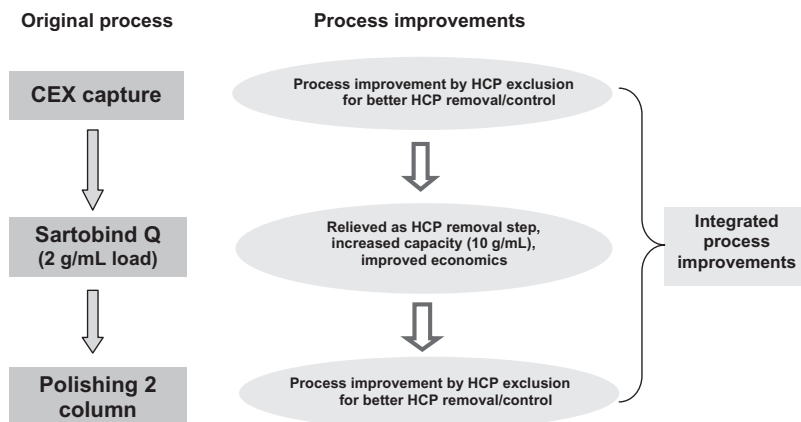


**FIGURE 5.6** Product and contaminant profiles during cation-exchange elution fractionation.



**FIGURE 5.7** CHOP clearance is influenced by elution pH as evidenced by HCIC elution pH gradient.

whereas reduction to 100ng/mg HCP can be achieved using a single separation step, yielding two-column ion-exchange processes. Resins like MEP with antibody selectivity can remove much larger HCP loads when tight exclusion criteria are established based on both pH (13) and peak characterization (Fig. 5.7).



**FIGURE 5.8** HCP exclusion strategy for integrated process improvements.

In nonaffinity purification schemes, a flow-through Q resin chromatography step can be replaced successfully by Q membrane chromatography (4, 25, 26). Using the HCP exclusion approach, process improvements could be made on adjacent chromatography steps leading to a higher loading capacity on the Q membrane. For example, in a three-column HuMab process, the Q membrane load capacity was enhanced from 2 to 10 g/mL by relieving the demand on HCP removal after adjusting the process conditions for CEX capture and another polishing step (Fig. 5.8). In this process, the Q membrane was utilized mainly to reduce the adventitious viral load, and with this improved loading capacity, the Q membrane was equal to or better than resin chromatography as shown by cost modeling (25–27). Thus, the HCP exclusion strategy enables integrated process improvements instead of just focusing on individual process steps.

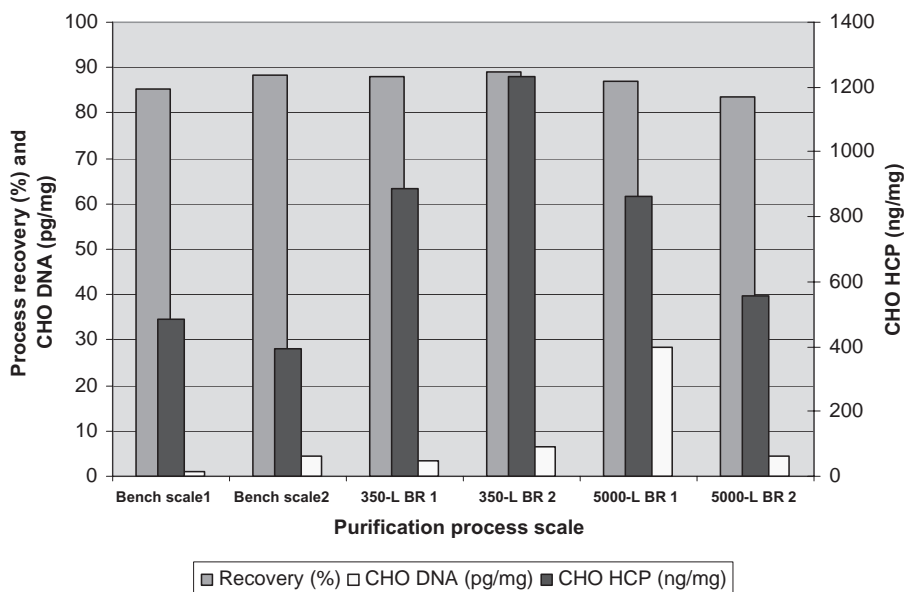
**5.3.3.2.4 Aggregates and Other Product-Related Contaminants.** High-molecular-weight species derived from the product are challenging impurities because they closely resemble the protein of interest. Because various multimers of the protein can form, with different sizes, they need to be reduced to the lowest acceptable levels specified for therapeutic-grade material (28, 29). Such species may be present in the cell culture supernatant, and their formation can be encouraged during low-pH purification steps, viral inactivation steps, and under conditions of high pressure and shear stress as employed during TFF.

Since affinity columns capture both monomers and aggregates, separation at the capture step is not feasible when using affinity-based processes. Purification can instead be achieved using ion-exchange chromatography processes based on different charge characteristics and/or hydrophobic interaction chromatography (see Chapter 6). For example, cation-exchange capture processes

can be optimized to reduce or to remove aggregates to the desired levels, thus improving purification at the capture stage. As a consequence, the demand on the subsequent polishing steps is reduced, enabling the development of simple, robust and scalable purification schemes.

**5.3.3.2.5 Nonaffinity Processes—Scalability and the Reuse of Capture Resins.** By implementing the above methodologies at different steps and thus excluding HCP gradually from the product, nonaffinity purification processes for various HuMabs have been developed successfully up to the 5000-L bioreactor scale (30). The process recovery, product purity, and contaminant removal profiles are highly comparable at different scales, proving the robustness of the manufacturing process (Fig. 5.9). Products from all these operation scales appear comparable following extensive analysis using a battery of physico-chemical and biological test methods and, for two HuMabs, extended characterization testing including peptide analysis and glycan profiling. Scale-down testing of cation-exchange processes has clearly demonstrated that the resin can be reused over 100 cycles for capture chromatography without affecting the efficiency of contaminant removal, product purity, or recovery (Table 5.8 and Fig. 5.10).

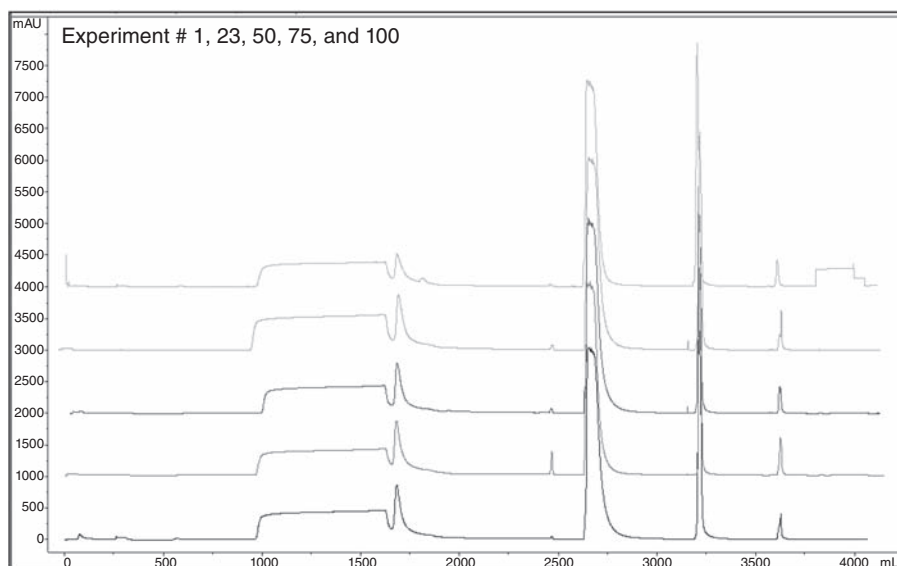
**5.3.3.3 Two-Column Nonaffinity Purification Processes.** Nonaffinity antibody separation processes also include at least one or two polishing steps to



**FIGURE 5.9** Process step recovery and contaminant removal profiles of cation-exchange capture at different process scales.

**TABLE 5.8 Consistency of Process Recovery, Product Purity, and Contaminant Removal in Cation Capture Recycling**

Cycle No.	Recovery, %	Product Purity, %	CHOP, ng/mg	CHO DNA, pg/mg
1	95.2	100	577	0.9
23	85.3	100	280	3.7
50	77.4	100	614	3.1
75	98.2	100	757	9.0
100	91.3	100	211	2.7

**FIGURE 5.10** Chromatographic performance of cation exchange as capture column for multiple cycles.

remove residual process and product-related contaminants, and the sequence of the steps appears to influence impurity levels (19). The replacement of one of the columns with a high-performance tangential flow filtration (HPTFF) step represents a significant advancement, which can reduce the host cell contaminants to acceptable levels in therapeutic-grade antibodies (31). It is more challenging to develop two-step purification processes using nonaffinity capture rather than affinity chromatography. However, the optimization of capture and polishing chromatography by monitoring and controlling HCP through the selective avoidance of certain process conditions can lead to the development of robust two-column processes for therapeutic-grade proteins. A flexible two-column nonaffinity scheme using cation exchange and HCIC has been developed and scaled up 10,000-fold for a variety of HuMabs (13). MEP Hypercel provides HuMab selectivity and can capture antibodies from

**TABLE 5.9 CHOP, DNA Levels and Virus LRV, in the Two-Step Purification Process Composed of Cation Exchange and HCIC**

Process Step	HuMab-1			HuMab-2		
	CHOP LRV	CHO DNA LRV	A-MuLV LRV	CHOP LRV	CHO DNA LRV	A-MuLV LRV
Cation-exchange chromatography	2.82	4.76	2.23	2.10	3.06	2.95
Low-pH treatment	n/a	n/a	5.03	n/a	n/a	>5.69
HCIC	0.97	1.30	5.15	1.35	1.20	>4.59
Virus nanofiltration	n/a	n/a	>4.92	n/a	n/a	>5.95
Total reduction	3.79	6.06	>17.33	3.46	4.26	>19.18
Minimum safety factor	n/a	n/a	7.46	n/a	n/a	9.85

n/a = not applicable. LRV: log reduction value; A-MuLV: amphotropic murine leukemia virus.

**TABLE 5.10 CHOP, DNA Levels and Virus LRV, in The Two-Step Ion-Exchange Purification Process Comprising Cation-Exchange and Anion Membrane Chromatography**

Product	HuMab-1			HuMab-2		
	CHOP, ng/mg	CHO DNA, pg/mg	A-MuLV LRV	CHOP, ng/mg	CHO DNA, pg/mg	A-MuLV LRV
Cation capture elution	21.9	39.5	2.01	593.7	15.8	2.39
Second anion membrane output	15.6	0.3	>5.75	38.0	<1.0	>5.66

high-ionic-strength feedstreams such as cell culture broths while providing the flexibility to function either as a capture or a polishing step (9). In addition, the significant reduction of adventitious viruses makes this resin desirable in simplified process schemes to yield therapeutic-grade proteins (Table 5.9).

Two-column purification processes based on ion-exchange offer further advantage by replacing flow-through mode chromatography with single-use membranes that are more operator-friendly (see Chapters 14 and 16). These designs achieve higher facility throughput, which is essential for handling high-titer cell culture processes, and they also offer the flexibility to allow integration into different facilities. A well-developed cation-exchange step in combination with anion-exchange chromatography offers a powerful separation scheme to remove HCP, DNA, aggregate, endotoxins, and viruses while achieving high product recovery rates for CHO platform technologies (Table 5.10) (2, 32, 33). They provide better process step integration strategies and

significantly reduce the cost of goods by alleviating the limitations imposed by traditional purification schemes.

Recently upgraded cation-exchange media achieve very high binding capacities of up to 140 mg/mL, which is four to five times higher and three to four times less expensive than currently marketed Protein A resins. By employing these high-binding resins as a capture step, 100-kg quantities of product can be handled by a two-step, nonaffinity process in a week, whereas traditional three-column purification schemes would have successfully handled only 2.5 kg at the same scale (Table 5.11) (see Chapter 10). Even better ion-exchange matrices that bind up to 250 g/L with fast processing times of 1000 cm/h (6) provide further potential to match the ever-increasing titers of cell cultures.

## 5.4 CONCLUSIONS

An integrated approach encompassing both upstream and downstream improvements can achieve the combined goals of reducing both the time to market and the cost of goods for biologics. To alleviate the constraints generated by existing downstream processes, purification technologies must be able to handle higher loads with faster throughput for commercial-scale antibody production (34). Process economics can be influenced by opting for simple purification processes using less expensive resins with higher binding capacities and fewer unit operations, without compromising the critical attributes of the product. Optimization of ion-exchange capture chromatography through the HCP exclusion strategy described above can simplify process designs, reducing the process to one column chromatography step and one membrane adsorption step, resulting in 20- to 40-fold improvements in facility throughput. Systematic optimization of capture by cation-exchange chromatography yields robust processes comparable in efficiency to Protein A affinity purification schemes, which can handle up to 5000 L of CHO cell culture broth. Comprehensive monitoring strategies for host cell contaminants must involve risk-based approaches from the early stages of cell culture development through to final downstream process designs, thus addressing quality by design aspects for process and product consistency that can be implemented eventually in process analytical technologies (35).

Disposable, single-use chromatography units add further value to ion-exchange schemes due to their simplicity, reduced validation costs, and lower capital investment (see Chapter 14). In addition, disposable process steps provide flexibility for existing facilities by processing larger batch volumes without significant facility modifications. Even processes involving potential alternative steps such as protein precipitation and crystallization can still exploit these disposable options for the efficient, high-throughput removal of trace contaminants (34, 36) (see Chapters 14 and 15). Furthermore, high-density and high-titer perfusion bioreactor processes can be integrated

TABLE 5.11 Increase in Downstream Throughput along with Upstream Improvement by Efficient Non-Protein A Process Schemes

Process Evolution	Old Process		Improved Process		Proposed Process				
Upstream: Expression level, g/L Production level (5000 L), kg Fold improvement in Upstream, X	0.5		5		10				
	2.5		25		50				
	—		10		20				
Downstream:  Purification process  Total days for process Total recovery % Total recovered product, kg Productivity, kg/d Fold improvement in downstream, X	Three-Column Process				Two-Step Process				
	DBC mg/mL	Step	Cycles	DBC mg/mL	Step	Cycles	DBC mg/mL	Step	Cycles
	15	CEX	2	100	CEX	2	140	CEX	3
		TFF	1	2100	AEX(M)	2	2100	AEX(M)	4
	20	AEX	1						
	13	Mixed mode	2						

Assume all chromatography steps use 1-m diameter column.  
Assume all membrane chromatography steps use 4 of 1620-mL module.  
Assume one cycle per day.  
DBC = dynamic binding capacity; CEX = cation exchange; TFF = tangential flow filtration; AEX(M) = anion exchange membrane.

efficiently with high-binding cation exchange-based capture steps and disposable polishing steps due to the desirable characteristics of the resin for multiple cycles, allowing continuous processing.

Since products are determined by the process in the protein therapeutics industry, time-tested chromatographic separations will continue to be used despite their throughput limitations until alternative technologies are thoroughly tested and demonstrated for process and product consistency (14, 37). Nonaffinity purification processes can bridge the gap between expensive affinity schemes and the more innovative processes for commercial biopharmaceutical production that have yet to be proven.

Efficient viral clearance strategies play a key role in the regulatory acceptance of two-step purification processes for antibody manufacture (38), and Q membrane chromatography certainly provides leverage here with its orthogonal mechanism of viral removal by adsorption in addition to low-pH inactivation and nanofiltration (see Chapter 8). For antibody production at the tone scale, virus removal filters can add substantially to process times, and careful evaluation and selection of filters for higher protein loading concentrations, capacities, and batch volumes are necessary to cope with ever-increasing antibody titers.

## 5.5 ACKNOWLEDGMENTS

Our thanks to Gisela Ferriera and the other downstream scientists who are contributing to the advancements of purification technologies, to Xiao-Ping Dai and Claudia Kloth for developing high-yielding cell culture processes, and to the entire process development team at Medarex.

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# 6

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## PURIFICATION OF MONOCLONAL ANTIBODIES BY MIXED-MODE CHROMATOGRAPHY

PETE GAGNON

### 6.1 INTRODUCTION

A Zen definition of mixed-mode chromatography might be “Mixed-mode chromatography is,” and that definition would be more accurate than any conceivable combination of technical terms. Strictly speaking, and whether intentional or not, every class of chromatography media exploits multiple modes of chemical interactions. Size-exclusion chromatography (SEC) media often bear charged groups and exhibit varying levels of hydrophobicity, either of which may significantly alter separation performance. Polymer backbone hydrophobicity and charges likewise alter the selectivity and capacity of ion-exchange (IEX) media. Spacer arms compound the issue—sulfopropyl cation-exchange (CEX) ligands have frequently been observed to offer different selectivities than less hydrophobic sulfoethyl ligands on the same solid phase. Indeed the whole field of hydrophobic interaction chromatography (HIC) arose from investigators discovering that alkyl spacer arms sometimes contributed more to solute binding than the affinity ligands they initially sought to investigate. Even bioaffinity chromatography is based on ligands that exploit well-defined combinations of discrete retention mechanisms. X-ray crystallographic analysis has shown that the interaction between Protein A and IgG is sterically aligned by the titration states of opposing histidyl residues at the interface, while the binding energy is dominantly hydrophobic with secondary

stabilization by four hydrogen bonds (1). Between the boundaries of bioaffinity and so-called single-mode chromatography methods resides a wide variety of synthetic ligands exploiting various combinations of protein-interactive chemistries. This chapter addresses antibody purification with some of the most promising of these synthetic mixed modes.

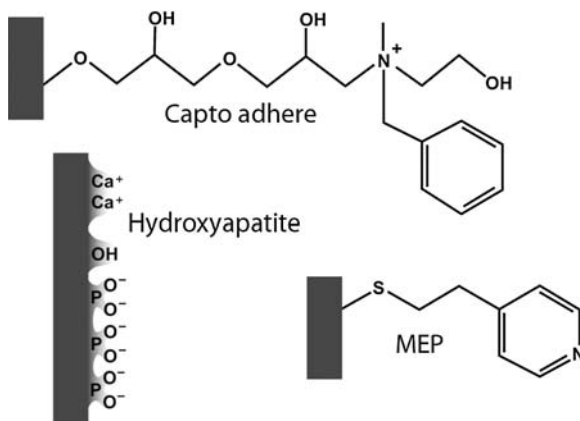
## 6.2 A BRIEF HISTORY

Mixed-mode retention mechanisms have been a part of chromatography since its origins. Hydroxyapatite (HA) was introduced in 1956 and was applied to antibody purification by no later than 1959 (2, 3). The advent of monoclonal antibodies (mAbs) triggered the development of many new mixed-mode ligands throughout the 1980s and 1990s. Notable examples included thiophilic chromatography (4–8), Avid AL (9, 10), and hydrophobic charge induction chromatography (HCIC) (11–15). Most of these were characterized by dominantly hydrophobic functionalities, accompanied by a variety of secondary functionalities intended to improve specificity. So-called biomimetic ligands began to appear in the mid-1990s (14–22). They were intended to provide a synthetic substitute for Protein A affinity chromatography (see Chapter 4). Recently introduced mixed modes such as Capto™ adhere and Capto MMC follow the lineage of charged/hydrophobic ligands but focus on polishing applications. Table 6.1 lists a selection of mixed-mode ligands and their retention mechanisms. Figure 6.1 illustrates the ligands discussed most in this chapter.

Despite promising indications and optimistic projections, the turn of the century found no commercial applications for mixed-mode chromatography in the purification of antibodies. Most suffered from some combination of low binding capacity, limited contaminant reduction, or poor antibody recovery. Meanwhile, media improvements and increasing sophistication in the use of IEX and Protein A affinity resins raised the level of performance necessary

**TABLE 6.1 Selected Mixed-Mode Ligands and Attributed Chemical Reactivities**

Ligand	Binding Mechanisms
HA	Metal affinity, CEX, hydrogen bonding (HB)
Fluorapatite	Metal affinity, CEX, HB
T-gel	Hydrophobic interaction (HI), thiophilic interaction (TI), HB
Avid-AL	HI, TI, HB, $\pi$ - $\pi$ bonding, anion exchange
MEP	HI, TI, $\pi$ - $\pi$ bonding, anion exchange
Capto MMC	HI, $\pi$ - $\pi$ bonding, HB, CEX
Capto adhere	HI, $\pi$ - $\pi$ bonding, HB, anion exchange
A1P	HI, HB, $\pi$ - $\pi$ bonding, anion exchange
A2P	HI, HB, $\pi$ - $\pi$ bonding, anion exchange
TG19318	HI, $\pi$ - $\pi$ bonding, anion exchange



**FIGURE 6.1** Structure of HA, MEP, and Capto adhere. The structural formula for hydroxyapatite is  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . Surface-accessible calcium residues occur in doublets called C sites, while accessible phosphoryl residues occur in triplets called P sites. The chemical name of MEP is 2-mercaptoethyl pyridine. The ligand for Capto adhere is N-benzyl-N-methyl ethanolamine.

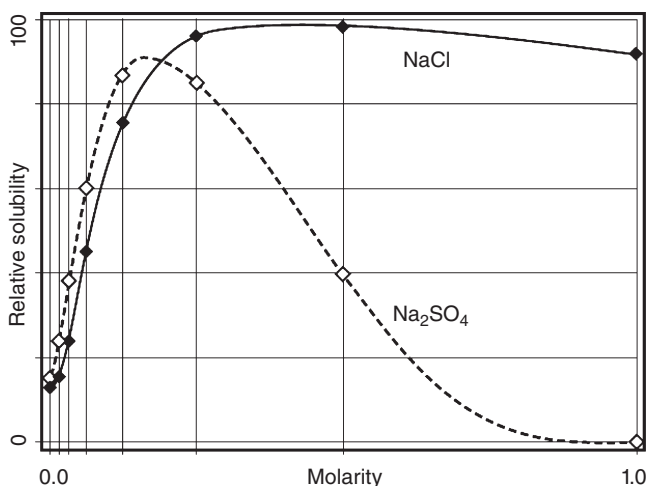
for mixed-mode to achieve a foothold. Concurrently, however, antibodies that were inadequately served by the standard purification tools were beginning to appear in various clinical portfolios. These notably included antibodies with high levels of aggregates. The 2003 publication of an HA method for aggregate removal—where all traditional methods had failed—signaled the turning of the tide (23).

### 6.3 PREREQUISITES FOR INDUSTRIAL IMPLEMENTATION

Resources are limited; stakes are high, and process developers are properly cautious about exploring methods that are not well understood. Establishing a mixed-mode method as an acceptable commercial processing option requires three steps: (1) the primary interactions of a mixed-mode ligand with antibody and major contaminant classes must be defined; (2) an efficient, preferably intuitive, approach to method development must be defined; and (3) leachate levels, removal methods, and potential toxicity must be characterized. HA is a good example of the importance of defining primary interactions. Its dominant retention mechanisms—phosphoryl CEX and calcium metal affinity—were described in the early 1980s, but the model solutes used in its characterization were generally impertinent to antibody purification (24–26). Widespread consideration of HA as a credible commercial tool did not begin until its interactions with IgG, aggregates, DNA, endotoxins, viruses, leached Protein A, and host cell protein (HCP) were published more than 20 years later (27, 28). With the exception of Capto adhere, similarly detailed

characterization for most charged/hydrophobic mixed modes is still lacking. Knowing the separation mechanisms for a given mixed mode provides a helpful starting point for method development, but complications arise when more than one of the component mechanisms responds to the same controlling variable. This is why the definition of a development pathway is essential. With HA for example, phosphate buffer simultaneously affects both the CEX and metal affinity mechanisms, making it impossible to control one without affecting the other. The situation is even more complex with charged/hydrophobic ligands, where the identity and concentration of salt are the polypotent variables. Insufficient salt concentration may reduce antibody solubility, potentially favoring nonspecific interactions and contributing to poor recoveries (29). Figure 6.2 shows the relative solubility of an IgG as a function of sodium chloride and sodium sulfate concentration. Solubility in the absence of either is about 80% lower than maximum solubility. Salt also suppresses charge interactions, including charge repulsion, which may be necessary for eluting IgG from charged/hydrophobic mixed modes. Even modestly higher salt concentrations may enhance hydrophobic interactions enough to retard product elution. The compound effect of these mutually antagonistic responses to a single process variable is a narrowing of the available design space, with the practical consequence of reducing the range of conditions within which selectivity can be optimized for the removal of key contaminants.

Any ligand that binds strongly to antibodies under physiological conditions poses a potential safety concern, and Protein A provides a model. Leached Protein A does not exist free in solution, but is complexed with IgG. If not removed during purification, it transfers to the patient's IgG upon administra-



**FIGURE 6.2** Effect of salt concentration on IgG solubility at pH 7.0. Results obtained with purified mouse monoclonal IgG2a (29).

tion (30). Protein A has been shown to mediate a wide range of immunological effects, hence the strong focus of regulatory authorities on its removal (31). In favor of Protein A, however, numerous methods for leachate removal have been developed; its toxicology profile has been characterized exhaustively, and Protein A-purified antibodies have compiled an enviable safety record. Leachate is not a concern with HA because it dissociates into calcium and phosphate ions. The Protein A mimetic TG19318 elicits no immunological response when conjugated to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA), and its intravenous (IV)  $LD_{50}$  values in mice are about 150 mg/kg (20). Other mixed-mode ligands will hopefully prove equally benign, but the issue needs to be addressed rigorously, especially given the suggestion that some are suitable as the final operation in two-step purification procedures. Placing them at the end of a process precludes the best opportunities for leachate removal. Consult suppliers directly for information on toxicology, leaching, and leachate removal.

#### 6.4 MECHANISMS, SCREENING, AND METHOD DEVELOPMENT

The interactions between HA and IgG and its major contaminants have been well defined. Most IgGs have weak affinity for HA calcium but have fairly strong charge interactions with HA phosphates. Setting a constant low level of phosphate suspends weak calcium affinity interactions but leaves strong ionic interactions intact. A sodium chloride gradient can then dissociate ionic bonds, which elutes monomeric IgG. Aggregates elute at higher sodium chloride concentrations. Contaminants with a strong calcium affinity remain bound until the column is cleaned with concentrated phosphate. These include leached Protein A–IgG complexes and phosphorylated contaminants such as DNA, endotoxin, and lipid-enveloped viruses (Table 6.2) (32, 33). Screening involves a series of runs exploiting sodium chloride gradients at constant low levels of

**TABLE 6.2 Summary of Contaminant Removal by HA. Data from NaCl Gradients at Constant Phosphate Concentrations<sup>a</sup>**

Contaminant	Assay Method	Clearance (LRV)
Aggregates	HPSEC	1–2
Protein A	Cygnus	1–2
CHOP	ELISA	2
DNA	Picogreen	>3
Endotoxin	LAL	>4
A-MuLV	Infectivity	>4
X-MuLV	Infectivity	>3
MVM	Infectivity	2

<sup>a</sup>References 23, 27, 32–35.

**TABLE 6.3 Conditions for Screening HA**

Materials and Processes	Description of Properties and Conditions
Media	Ceramic HA, CHT™ type I, 40μm
Buffer A	50mM MES, 5mM sodium phosphate, pH 6.5
Buffer B	50mM MES, 5mM sodium phosphate, 2M sodium chloride, pH 6.5
Buffer C	500mM sodium phosphate, pH ≥ 6.5
Flow rate	200–300cm/h
Equilibrate column	Buffer A
Inject	Protein A-purified IgG
Wash	5 column volumes (CV), buffer A
Elute	30 CV linear gradient to buffer B
Clean	Buffer C

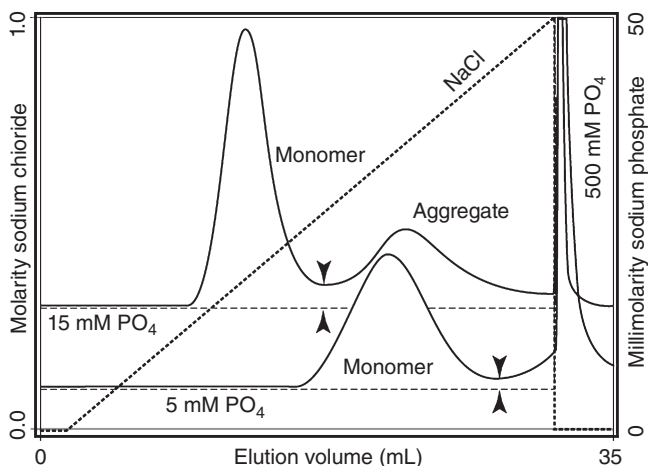
*Note:* If antibody fails to elute within the sodium chloride gradient, increase the phosphate concentration in buffers A and B to 10mM and repeat. If the antibody still fails to elute, increase phosphate concentration to 15mM, etc. Optimization may also include evaluation of pH values up to 7.5. A similar approach may be employed with ceramic fluorapatite (CFT™, type II, 40μm), but the pH may be reduced to 5.5.

phosphate (Table 6.3). In general, the best aggregate separation is obtained with the lowest phosphate concentration at which nonaggregated antibody elutes in sodium chloride. High throughput screening and statistical design of experiments (DoEs) have also been applied to HA, with dramatic success: aggregate reduction at the industrial scale from more than 60% to less than 0.1% (34).

An attractive feature of HA is that early method development can be monitored by visual inspection of the separation between IgG monomer and aggregate peaks on the chromatogram (Fig. 6.3). This approach is enabled by the fact that DNA, endotoxins, leached Protein A, and viruses elute in the high-phosphate cleaning step, long after the antibody has been eluted. This makes optimization of their removal essentially a by-product of optimizing aggregate removal. Expensive and time-consuming secondary tests for aggregates, HCP, and leached Protein A can be deferred until after conditions supporting the best aggregate fractionation have been identified. HA can be cleaned effectively with 500–600mM phosphate and is stable in 1M sodium hydroxide for at least 20months (32). It may be stored in 0.1M NaOH or 20% ethanol plus 5mM sodium phosphate, pH ~7.0.

Another attractive feature of HA is that the majority of antibodies behave fairly consistently, enabling a platform approach to aggregate removal. The main exception concerns antibodies that require phosphate concentrations greater than about 20mM to achieve elution in a sodium chloride gradient. Polyethylene glycol (PEG) has been shown to enhance aggregate resolution well beyond baseline separation, regardless of the phosphate or chloride concentrations required to elute a particular mAb (Fig. 6.4) (35). PEG is a component of many Food and Drug Administration (FDA)-approved injectable



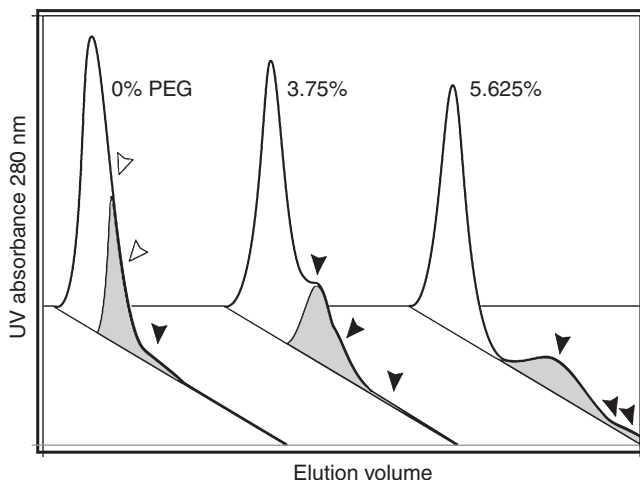


**FIGURE 6.3** Aggregate separation on HA as a function of phosphate concentration. CHT type I, 20  $\mu\text{m}$ , 7.5 mm  $\times$  5 cm, 300 cm/h; buffer A, 5 or 15 mM sodium phosphate as indicated, pH 6.5; buffer B, 1 M sodium chloride in 5 or 15 mM phosphate as indicated, pH 6.5; buffer C, 500 mM sodium phosphate, pH 6.5. Equilibrate column in buffer A; inject 100  $\mu\text{L}$  Protein A-purified monoclonal IgG1 chimera; wash in five column volumes (CVs) buffer A; elute in 30 CV linear gradient to 100% buffer B; clean with buffer C. Note that the separation between monomer and aggregate comes nearer to baseline at the lower phosphate concentration (see arrows).

product formulations (36). It also enhances aggregate separation on ion exchangers and can reasonably be expected to do so with other mixed modes (35).

A minor compromise with HA is that operating pH must be kept above pH 6.5 and a minimum concentration of 5 mM phosphate must be present to maintain matrix stability. Exposure to chelating agents, including citrate, must be avoided. Yellow-reddish brown discoloration is sometimes observed at the top of an HA column, indicating an uncontrolled source of metal contamination, usually iron. The appropriate remedy for this observation is elimination of the metal contamination source, since metals bind to proteins and can alter their charge, hydrophobicity, and functional properties (31, 37–39). In this sense, the ability of HA to remove metals is beneficial to the product, and is unique among the purification methods commonly used for commercial antibody purification. HA has a reputation for being difficult to pack, in part because of its high density and rapid settling rate, and in part because the particles can be damaged by inappropriate handling, usually during unpacking. Large-scale column designs using dynamic axial compression make packing—and repacking—as simple and reproducible as with any other chromatography media.

Charged hydrophobic mixed modes behave differently in every respect, as illustrated by immobilized 2-mercaptoethyl pyridine (MEP HyperCel™,



**FIGURE 6.4** Effect of PEG on aggregate resolution with HA. CHT type I, 20  $\mu$ m, 5 mm  $\times$  5 cm, 300 cm/h (1 mL/min); buffer A, 10 mM sodium phosphate, plus the indicated % PEG-4600, pH 6.5; buffer B, 250 mM phosphate, plus the indicated % PEG-4600, pH 6.5; buffer C, 500 mM sodium phosphate, pH 6.5. Equilibrate column in buffer A; inject 100  $\mu$ L Protein A-purified monoclonal IgG1 chimera; wash in five CVs buffer A; elute in 20 CV linear gradient to 100% buffer B; clean with buffer C. This is the same antibody illustrated in Fig. 6.3, but is eluted in a phosphate gradient. No aggregate resolution was apparent in the absence of PEG, but the separation at ~5% PEG was better than the best results obtained with sodium chloride gradients at constant low-phosphate concentration. Arrows indicate aggregate populations.

Fig. 6.1) (12). At neutral pH, MEP acts primarily as an HIC ligand. High ligand density supports binding at physiological salt concentration. A sulfur moiety in the spacer arm is intended to confer thiophilic affinity but experimental data suggest that its contribution is nil (14). The mixed-mode character of MEP begins to emerge with decreasing pH as the nitrogen in the pyridyl ring becomes charged, hence the name hydrophobic charge induction. Decreasing pH simultaneously increases the positive charge on protein amino residues, inducing electrostatic repulsion between the ligand and the antibody. This overcomes the remaining hydrophobic interaction, leading to antibody elution. Interactions with the various contaminant classes have not been fully defined. Most HCPs flow through but many are retained with the antibody. Aggregates tend to elute after nonaggregated antibody. Leached Protein A should behave likewise, but this remains to be confirmed. Most DNA should flow through since MEP is uncharged at neutral pH. As noted above, the effects of salt on antibody elution are polypotent, and a standardized approach to method development that accommodates the range of charge and hydrophobic variation among mAbs has yet to emerge. Screening pH gradients over a range of salt concentrations may be useful to identify the overall dimensions of the design space for a given mAb (Table 6.4).

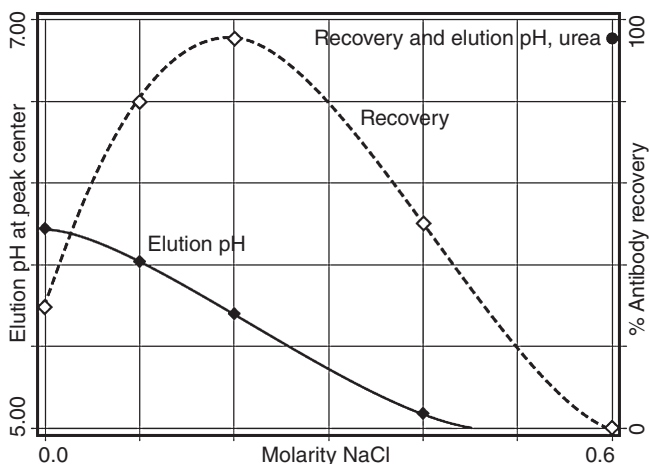
**TABLE 6.4 pH Gradient Screening Conditions for Charged/Hydrophobic Mixed Modes**

Materials and Processes	Description of Properties and Conditions
Media	MEP, Capto adhere, or other positively charged/hydrophobic ligand
Buffer A	10mM sodium citrate, 10mM sodium phosphate, pH 7.2
Buffer B	10mM sodium citrate, 10mM sodium phosphate, pH 4.0
Buffer C	2M guanidine, pH 5.0
Flow rate	300cm/h or per manufacturer's recommendations
Equilibrate column	Buffer A
Inject	Protein A-purified IgG
Wash	5 column volumes (CVs), buffer A
Elute	15 CV linear gradient to buffer B
Clean	Buffer C

*Note:* In a second run, add sodium chloride to buffers A and B, to a final concentration of 0.1 M. In a third run, add sodium chloride to buffers A and B, to a final concentration of 0.2 M, etc., until the antibody recovery maxima have been detected, as indicated by conditions under which the largest proportion of antibody (elution plus cleaning peak) elutes within the pH gradient. A similar approach may be employed with negatively charged mixed modes such as Capto MMC, except reversing buffers A and B. Note that these conditions may be different from those recommended by suppliers.

Within the pH ranges used for traditional AEX and CEX applications, antibodies generally will not elute in salt gradients from either Capto adhere (Q/phenyl) or Capto MMC (carboxy/phenyl). Electrostatic repulsion is necessary for elution, with decreasing pH on Capto adhere, and with increasing pH on Capto MMC. Correlation of elution behavior with isoelectric point (pI) is confounded by hydrophobic interactions. This is illustrated in Fig. 6.5, where the pH at IgG peak center in a pH gradient decreases more or less linearly from 5.84 to 5.04 vs. an increase in sodium chloride concentration from 0.0 to 0.4 M. This antibody, with a pI spanning the range 7.8–8.2, failed to elute at any pH in 0.6 M NaCl, until the experiment was repeated in the presence of 2 M urea, at which point it eluted at pH 6.78. This behavior is more typical of conventional ion exchangers and suggests that the urea suspended the hydrophobic component of antibody binding. It may also reflect suspension of hydrogen bonds, since urea is both a strong hydrogen donor and acceptor (40).

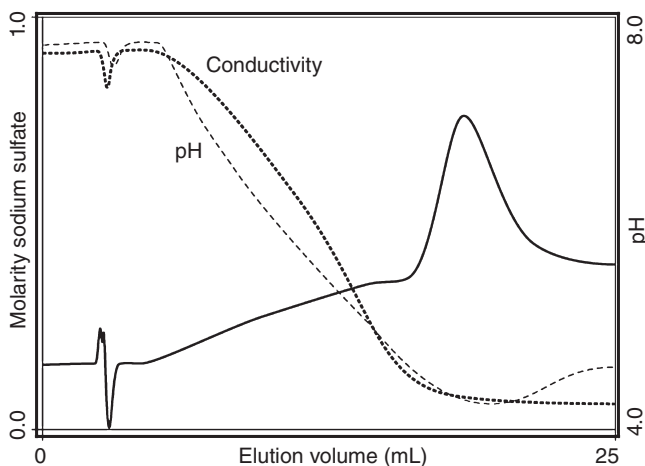
Figure 6.5 also plots the recovery of the same antibody as a function of sodium chloride concentration. The plot is strongly reminiscent of the salting-in/salting-out curve of IgG vs. sodium sulfate, a strong precipitating salt (illustrated in Fig. 6.2). This behavior is consistent with a strong hydrophobic contribution by the ligand. When the experiment at 0.6 M NaCl was repeated in the presence of 2 M urea, recovery was about 95%. These data explain why Capto adhere is able to achieve good binding even at high salt concentrations: the hydrophobic interaction mechanism compensates for the loss of AEX



**FIGURE 6.5** The effect of sodium chloride on IgG elution pH and recovery from Capto adhere. Capto adhere, 1 mL HiTrap™, 1 mL/min; buffer A, 10 mM sodium citrate, 10 mM sodium phosphate, plus the indicated concentration of sodium chloride, pH 7.5; buffer B, 10 mM sodium citrate, 10 mM sodium phosphate, plus the indicated concentration of sodium chloride, pH 4.5. Equilibrate in buffer A; inject 100  $\mu$ L Protein A-purified IgG1 chimera; wash in buffer A; elute in 15 CV linear gradient to 100% buffer B. Elution pH indicates the pH value at IgG peak center. Recovery indicates the proportion of the antibody eluting within the gradient. The experiment at 0.6 M sodium chloride was repeated with 2 M urea added to both buffers; results at upper right.

binding. This is highlighted by effective antibody binding even in 0.9 M sodium sulfate (Fig. 6.6) and demonstrates that in addition to tolerating a wide range of conductivities in Protein A eluates, Capto adhere can accommodate high-salt samples from intermediate CEX, HIC, or HA purification steps. Due likewise to its strong hydrophobic component, MEP also supports sample loading over a wide range of salt concentrations (12–14, 41).

Translating data from a limited number of bind-elute experiments to a preliminary definition of design space for a flow-through application is complicated by a discrepancy between binding and elution isotherms. Proteins will often flow through a column under conditions that would be insufficient to cause the same protein to elute if bound. This applies to all chromatography methods and is referred to, in polite company, as hysteresis. Its practical significance is that process developers can consider a wider range of flow-through conditions than may be indicated by bind-elute results. With the antibody discussed above, for example, the design space might be defined by pH values ranging from about 5.5 to 7.5, and conductivity values ranging from about 10 to 30 mS/cm. Once the boundaries of the design space have been identified, DoE has been applied with Capto adhere to identify conditions that satisfy regulatory requirements for all major contaminant classes (42, 43) (Table 6.5). Capto adhere supports about 5.8 log reduction of minute virus of mice (MVM)



**FIGURE 6.6** Antibody binding on Capto adhere in concentrated salt. Capto adhere, 1 mL HiTrap, 1 mL/min; buffer A, 10 mM sodium citrate, 10 mM sodium phosphate, 0.9 M sodium sulfate, pH 7.5; buffer B, 10 mM sodium citrate, 10 mM sodium phosphate, pH 4.5. Equilibrate in buffer A; inject 100  $\mu$ L Protein A-purified IgG1 chimera; wash in buffer A; elute in 15 CV linear gradient to 100% buffer B. The uneven baseline is from differential UV absorption by the A and B buffers. This antibody failed to elute when pH was maintained at 7.5 (data not shown).

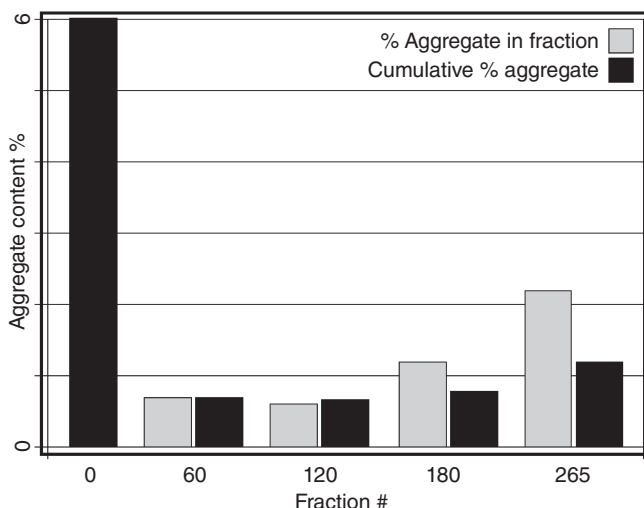
**TABLE 6.5 Summary of Purification Performance for Capto Adhere**

mAb	Load pH	Load Conductivity	Recovery, %	Aggregate, %	HCP, ppm
1	7.0	8	90	0.5	<15.0
2	5.5	3	95	0.6	2.0
3	6.0	2	95	0.8	9.0
4	7.0	20	91	0.2	30.0
5	7.5	20	92	<0.1	7.5

Source: All data obtained in flow-through mode (42, 43).

at conductivities ranging from 10 to 30 mS, whereas reduction of murine leukemia virus (MuLV) declines from 4.5 to 3.6 logs over the same range (43). In general, high pH and low conductivity favor the most effective removal of HCP and leached Protein A.

Preliminary data indicate that aggregate removal is also most effective at high pH, but is relatively independent of conductivity up to about 50 mS (43). Antibody recovery appears to be optimal at acidic pH and at more moderate conductivity values, essentially opposite to the conditions offering the best contaminant clearance. This correctly implies that antibody loading is an important determinant of overall performance (Fig. 6.7). As with most methods, aggregate removal is likely to be the limiting factor, which suggests that initial



**FIGURE 6.7** Aggregate reduction by Capto adhere. Fraction 0 represents the starting material: Protein A-purified IgG (43).

method development can focus on this parameter. Although aggregates elute after the nonaggregated antibody, they do not typically elute in a distinct peak. This means that analytical SEC will probably be required to evaluate results even in early development stages, although assays for leached Protein A and HCP can perhaps be deferred until the conditions for aggregate removal have been established.

Current product literature for Capto adhere recommends that specific cleaning conditions be developed for each process according to the type of contaminants present. The media can withstand exposure to 1M NaOH (1 week at 40°C), 1M acetic acid, 70% ethanol, and all commonly used aqueous buffers. Exposure to oxidizing agents or anionic detergents should be avoided (43). Recommendations for the development of specific cleaning conditions and the avoidance of anionic detergents are a subtle warning that removing strongly hydrophobic negatively charged compounds may be a challenge. This is highlighted by the difficulty of removing DNA from traditional Q anion exchangers. Even combinations of 2M NaCl and 1M NaOH have proven insufficient to achieve mass balance (44, 45). This is because Q anion exchangers retain a positive charge even in NaOH. High conductivity is insufficient for elution because of the multipoint interactions between the exchanger and the many phosphoryl residues on DNA. It seems reasonable to expect that this challenge should carry over to Q-containing mixed modes, and may be compounded by the additional hydrophobic interaction. In the experiments described in Fig. 6.5, urea has been shown to weaken the hydrophobic component of Capto adhere, and suggests that compounds like urea, guanidine, and other chaotropes may prove useful as cleaning agents.

Biomimetic ligands represent the most complex mixed modes, but also those with operational characteristics most similar to Protein A. The peptide mimetic TG19318 is based on a tetradentate lysine core with identical Arg-Thr-Tyr additions to each of the arms (20). TG19318 binds IgG from all subclasses, plus IgA, IgE, IgM, and IgY (see Chapter 18). IgG-binding capacities up to 25 mg/mL have been reported, with albumin being the major contaminant (17, 20). Another lineage of Protein A mimetics has employed a tridentate triazine as a scaffold, with anilino and tyramino substitutions to mimic the properties of a Phe-Tyr dipeptide shown to be an important component of the interaction between Protein A and Fc (46). The ligand A2P, a member of this lineage, binds all IgG subclasses from many mammalian species. Recent data obtained with mAbs in Chinese hamster ovary (CHO) cell culture supernatants confirmed its capture abilities, but HCP clearance and antibody recovery were significantly lower than achieved by Protein A (14). Both TG19318 and A2P can be eluted with a step to low pH. As with simpler charged/hydrophobic mixed modes, experimentation with pH gradients over a range of conductivities may reveal conditions that offer better purification and recovery.

## 6.5 CAPTURE APPLICATIONS

The relative economy and sodium hydroxide resistance of mixed-mode ligands has predictably led to virtually all of them being evaluated as alternatives to Protein A. HA achieves purity comparable to Protein A and has the advantage of removing aggregates in tandem, but the conductivity of cell culture supernatants generally depresses its IgG-binding capacity to a noncompetitive level, and compensation by dilution or diafiltration are both unattractive (47, 48). Fouling is also a concern since supernatants typically contain significant amounts of iron, phospholipids, and DNA, all of which bind strongly to HA. Chelating agents in cell supernatants may cause HA degradation.

Charged/hydrophobic mixed modes with a strong AEX functionality are unlikely to evolve as capture options because of their strong interactions with phospholipids and DNA. This is likely to reduce IgG capacity significantly in both flow-through and bind-elute modes. As noted above, however, the key issue is cleanability.

MEP bypasses these problems because it is uncharged at neutral pH, and recent data suggest that it may be commercially viable as a capture step (Fig. 6.5) (41, 49–51). Binding capacities for mAbs are 17–30 mg/mL. Recovery is about 5% lower than Protein A, and elimination of HCP is about 99% compared to 99.9% on Protein A (49, 51). Aggregate clearance, however, is better by factors ranging from 1.6 to 4.2-fold; MEP is much cheaper than Protein A, and there is no Protein A leachate to eliminate. Other recent studies have highlighted these same points and have documented effective IgG capture but lower clearance of HCP (14, 52). The discrepancy may relate to diversity among antibodies, composition of cell culture supernatants, or separation

conditions. As with Protein A, a second wash with some combination of elevated sodium chloride, ethylenediaminetetraacetic acid (EDTA), arginine or urea to weaken hydrophobic interactions, and reduced pH (but not enough to elute IgG) may be useful to enhance HCP clearance prior to elution.

Negatively charged hydrophobic mixed modes have also been evaluated as capture alternatives. Column loading requires a modest reduction of feed-stream pH but tolerates physiological conductivity. Purity is good, though not equivalent to Protein A. Capacity is also lower than Protein A, but large-scale industrial feasibility has been documented by commercial purification of a polyclonal bovine IgG from milk products (53). Performance and variability with mAbs remain to be demonstrated.

The biomimetic ligand A2P recovered polyclonal IgG from the serum of hyperimmunized sheep at a purity of 85%, but has not yet been exploited commercially for the purification of mAbs (54).

## 6.6 POLISHING APPLICATIONS

Sodium chloride elution from HA provides a broadly applicable option for removing double-digit percentages of aggregates from Protein A-purified IgG. Recent presentations have demonstrated its ability to reduce aggregate loads in the 40–60% range, down to less than 0.1% (32–34). PEG promotes equally or more effective aggregate removal than sodium chloride but imposes the complication of removing residual PEG (35). HA is also effective for removing antibody fragments. Dynamic IgG-binding capacities range from 20 to 60 mg/mL. Feasibility has been demonstrated for two-step purifications with Protein A and HA, but some HCPs may elute before the antibody, suggesting that HA should provide its best results in bind-elute mode, at least in two-step procedures. Preliminary data suggest that aggregate resolution may be better on fluorapatite, which exploits the same mechanisms as HA, but its capacity appears to be lower (33).

As noted above, MEP and Capto adhere have both demonstrated a potential for aggregate removal. Two recently published examples describe aggregate reduction by Capto adhere in flow-through mode from 0.7% to less than 0.1%, and reduction from 6% to about 0.8% with 180 mg/mL of IgG per gel load, or to about 1.2% with a 265 mg/mL load (Fig. 6.7). Final aggregate concentrations for antibodies with unspecified initial aggregate loads range between these values (Table 6.5). Capto adhere is also effective for the removal of antibody fragments (43). Another attractive feature of Capto adhere is that it has been designed for flow-through applications. As with other adsorption chromatography methods, however, it can reasonably be expected to support more effective contaminant clearance when employed in bind-elute mode. This may not be necessary in the context of three-step antibody purification procedures, but it may extend the range of antibodies that can be purified effectively with two-step procedures. This seems a modest sacrifice for the



opportunity to eliminate an intermediate process step—which itself is likely to be a bind-elute step—plus all the accompanying expense of materials, equipment, method development, and validation. These observations apply equally to other mixed modes that may support two-step purification procedures.

## 6.7 SEQUENTIAL CAPTURE/POLISHING APPLICATIONS

Recent presentations and publications have documented the ability of multi-step mixed-mode procedures to achieve purification without recourse to either Protein A or conventional ion exchangers (49–51). Two-step procedures comprising MEP with HA, while offering respectable performance, have not demonstrated adequate contaminant clearance to support commercial applications (49). A three-step procedure comprising MEP, Capto adhere, and HA has cleared this hurdle and offers worthy economic and regulatory benefits (51). Such applications seem unlikely to threaten the dominance of Protein A and IEX in the foreseeable future, but they signal a clear intent to do so at the earliest opportunity.

## 6.8 THE FUTURE

The success to date of mixed-mode chromatography methods has been predicated on their ability to achieve purification process design goals for which traditional methods fail, i.e., aggregate removal. Future inroads will likewise require the demonstration of significant superiority over traditional methods. The pharmaceutical industry is conservative, and equivalent function, though promising, is not adequate rationale to justify the risk and expense of implementing a new technology.

The evolution of mixed modes seems likely to follow two complementary pathways. The first involves the development of buffer systems that support more effective fractionation. The recent discovery that PEG enhances aggregate separation with a wide variety of separation methods indicates that more remains to be learned, and new developments can be expected. It is tempting to speculate that buffer developments are unlikely to yield radically new capabilities, but this is exactly what has happened with sodium chloride and aggregate removal on HA, and the dearth of attention paid so far to the potential contributions of different Hofmeister salts leaves the door wide open for all types of mixed modes.

The second pathway—the one that would seem more likely to yield radical new capabilities—involves the evolution of mixed-mode ligands themselves. Continuing valuable but incremental improvements can be expected with simple ligands like apatites and charged/hydrophobic mixed modes, but the fact that they have taken this long to come this far suggests that another

dimension of functionality may be required to bring about a major paradigm shift. One of the reasons Protein A is so effective is that its interaction with IgG involves an induced fit (1, 14, 30). To its discredit, the induced fit is achieved by the destabilization of the C $\gamma_2$  domain of the IgG, but perhaps this offers guidance for a new direction in the development of mixed modes: if a mixed mode that participates in an induced fit interaction can be designed, but with the conformational change restricted to the ligand, mixed-mode chromatography might finally be in position to fundamentally alter the way the industry purifies antibodies.

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# INTEGRATED POLISHING STEPS FOR MONOCLONAL ANTIBODY PURIFICATION

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## 7.1 INTRODUCTION

Monoclonal antibodies (mAbs) have become the most important therapeutic modality in the biotechnology industry (1, 2). There are 21 Food and Drug Administration (FDA)-approved antibodies currently on the market, with many more under development in pharmaceutical pipelines. Given the high dose requirement and the increasing market potential of this therapeutic class, the primary focus during process development is to reduce manufacturing costs, streamline process development activities, and enable production at very large scales (3, 4).

Antibodies are typically produced using cultured mammalian cells to ensure proper folding and glycosylation. Over the last decade, significant advances have been made in cell culture technology, including the improvement of production media and feeding strategies, resulting in very high cell culture titers ( $>5$  g/L) (5). The efficient recovery and purification of antibodies from cell culture media is a critical part of the antibody production process (6). One of the recent trends in therapeutic antibody downstream processing is the adoption of templated purification schemes to enable shorter development times and multiproduct harmonization at manufacturing scales. This platform approach has been widely adopted by almost all companies with significant numbers of antibody candidates in their development pipelines. The approach

hinges predominantly on the successful use of Protein A affinity chromatography as the capture step, a highly selective process that can result in >95% purity starting from complex cell culture media (see Chapter 4). Following the Protein A step, there typically remain trace levels of process-related contaminants [such as host cell proteins (HCPs), DNA, leached Protein A, endotoxins, and some cell culture media additives] as well as product-related impurities (such as higher-molecular-weight aggregates and lower-molecular-weight degradation products). The subsequent chromatography processes are commonly referred to as the polishing steps since they remove trace-level impurities and serve to reduce these impurities to levels that assure product safety.

Various modes of chromatography including cation exchange (CEX), anion exchange (AEX), hydrophobic interaction chromatography (HIC), and hydroxyapatite (HA) have been used as polishing steps in antibody purification processes (7, 8). The use of immobilized metal-chelate affinity chromatography (IMAC) and size-exclusion chromatography (SEC), although less common, has also been reported (7, 9). Genentech has developed a generic purification scheme involving CEX followed by AEX in flow-through mode as the polishing steps after Protein A chromatography (10, 11). On the other hand, Amgen has adopted the use of a flexible downstream platform to account for biochemical differences seen among various antibodies (12). In this approach, two polishing steps are typically chosen from the four common modes listed above. Furthermore, Wyeth BioPharma has discussed the possibility of a two-step purification platform that employs a single polishing step (13). Typically, the organization of the polishing steps (number and mode) is case specific and is dictated mainly by the predominant impurities found in the respective downstream processes.

This chapter presents an overview of the most frequently used polishing steps in antibody manufacture and discusses the considerations to be kept in mind while integrating these steps into the overall purification scheme.

## **7.2 POLISHING STEPS IN ANTIBODY PURIFICATION**

### **7.2.1 Ion-Exchange (IEX) Chromatography**

IEX chromatography is widely used in the biopharmaceutical industry for the process-scale purification of monoclonal antibodies, fusion proteins, and other protein therapeutics (14–17). IEX is predominantly based on electrostatic interactions between surface charges on proteins and charged functional groups on the chromatographic resin. Differential adsorption/desorption separates the product from impurities (see Chapter 5). The protein-binding characteristics are determined by net charge of the protein and the charge distribution over the surface as well as the resin type (negatively charged for CEX chromatography or positively charged for AEX chromatography), ligand type (strong or weak ion exchanger), functional group, ligand density, base



matrix, linker chemistry, and pore size. IEX is the best-characterized chromatography mode. The stoichiometric displacement model (SDM) (18) and steric mass action (SMA) model (19, 20) provide a basic understanding of the adsorption phenomena in IEX, while a more complete description of the process has been described by Stahlberg (21).

**7.2.1.1 AEX Chromatography.** AEX chromatography is very popular for polishing in antibody manufacture because the high pI of most human antibodies prevents them from binding to AEX resins under the pH conditions (pH 7.0–8.0) typical for AEX. Higher pH conditions can increase the capacity for antibody binding, but this is usually avoided to minimize the risk of deamidation and proteolysis (8). On the other hand, impurities such as DNA, HCP, and endotoxins are negatively charged and thus bind strongly to AEX columns under the same conditions. It has therefore become common practice to operate the AEX step in flow-through mode under alkaline pH conditions and low conductivity. AEX flow-through chromatography also achieves excellent virus removal and has been validated as a generic step for virus clearance (22). However, the AEX step does little to eliminate aggregates and leached Protein A, leaving the burden of their removal for other polishing steps. The contaminants that are present after Protein A affinity chromatography are in trace amounts (typically ppm or ppb), thereby enabling the AEX flow-through step to be carried out at loading capacities of up to 100 mg/mL of resin. Table 7.1 lists some of the commonly used resins for this mode of chromatography.

Membrane chromatography is becoming a viable alternative to AEX chromatography in flow-through mode (23–26). Traditionally, one of the major disadvantages of membrane chromatography is the low binding capacity reflecting the lower surface area-to-bed volume ratio (27, 28). Those hurdles are circumvented by operating in flow-through mode, where only trace quantities of contaminants need to bind.

It is sometimes prudent to screen AEX in binding as well as in flow-through mode, since surface charge distributions may enable the binding of antibodies to AEX resins under appropriate solution conditions. Recently, it has been reported that the AEX step can be operated in an isocratic mode referred to as weak-partitioning chromatography (WPC) (29). In this mode, appropriate solution conditions are chosen to allow for significant product binding (1–20 g/L of resin). However, since the antibodies are more basic than the other impurities, the impurities bind even more strongly to the resin and act by sample displacement. Despite significant levels of product binding to the resin, high yields (>95%) can be achieved by using high loading (up to 250 g/L of resin) and short isocratic washes. Operating the AEX step in this WPC mode preserves the isocratic operation typical of AEX flow-through steps while providing greater selectivity for impurity removal. Using this as the only polishing step, significant impurity clearance has been reported including a log reduction value (LRV) of ~4 for HCP, >2 for leached Protein A, >3 for nucleic acid, >5 for retroviruses, as well as a ~20-fold reduction in product aggregates (13).



TABLE 7.1 Commonly Used AEX Resins

Resin Name	Vendor	Ligand	Base Matrix	Average Particle Size, $\mu\text{m}$	Pore Size, $\text{\AA}$
Super Q 650 S, M, and C	Tosoh	Quaternary ammonium	Methacrylate	35, 65, 100	1000
Q Sepharose FF	GE	Quaternary ammonium	6% cross-linked agarose	90	n/a
Capto Q	Healthcare		Highly cross-linked agarose with dextran surface extender		2000
Q Sepharose XL			6% cross-linked agarose with dextran surface extender		n/a
Fractogel EMD	Merck KGaA	Trimethyl ammonium ethyl	Methacrylate resin with polymeric "tentacles"	65	800
TMAE (M), Hicap					
Unosphere Q	Bio-Rad	Quaternary ammonium	Polymeric	120	n/a
Q Ceramic Hyper D	Pall	Quaternary ammonium	Ceramic bead filled with a hydrogel	50	n/a
POROS HQ50	Applied Biosystems	Quaternized polyethyleneimine	Coated cross-linked poly(styrenedivinylbenzene)	50	1600

n/a = not applicable.

**7.2.1.2 CEX Chromatography.** CEX chromatography is also used frequently for polishing in antibody manufacture (12, 30, 31), although in this case, the high pI of most human antibodies means that the step is run in bind-and-elute mode with high loading capacities and good selectivity. CEX has been shown to clear HCP and leached Protein A, as well high-molecular-weight (HMW) aggregates in specific cases (7). CEX is more effective than AEX in reducing leached Protein A levels. Protein A is relatively acidic and is thus retained less strongly than antibodies, leading to removal in the flow-through or through an intermediate pH wash. Fragments of leached Protein A that bind to an antibody can also be removed by an intermediate pH wash.

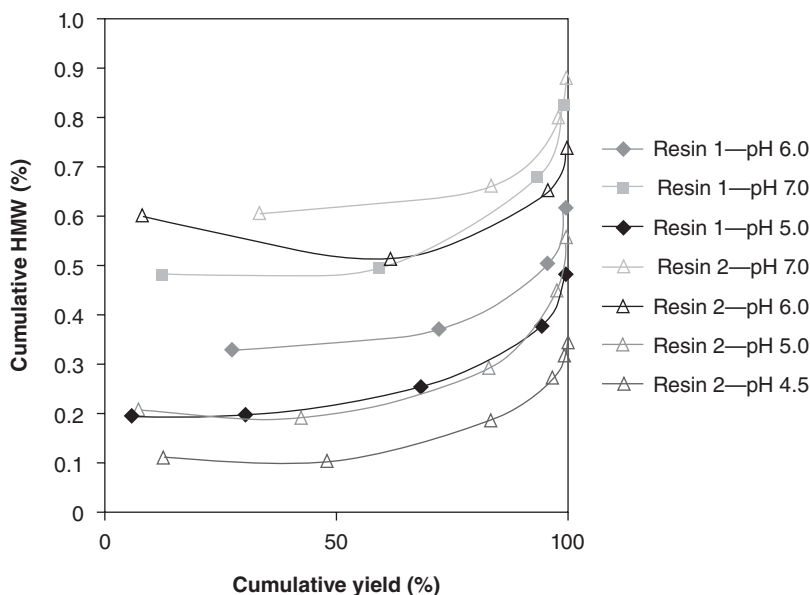
Table 7.2 lists some of the strong CEX resins commonly used in antibody manufacture. Weak cation exchangers such as carboxymethyl resins, although less frequently used, are also available from the listed vendors. Resins with a polymeric backbone such as methacrylate have greater selectivity for aggregate removal due to nonspecific hydrophobic interactions with the backbone (32). Knudsen and colleagues (23) have also evaluated CEX membranes for process-scale antibody purification and concluded that they might not be economical in a bind-and-elute mode.

The choice of CEX resin and the optimization of operating conditions depend largely on the nature of the impurities to be removed and the antibody of interest. Most antibodies are basic, yet they can vary significantly in their retention on CEX resins (12). Although one study has argued the possibility of predicting CEX retention behavior of human monoclonal antibodies based on amino acid sequence in the variable heavy chain (VH) region (33), in practice, process development largely relies on systematic experiments to select the appropriate resin and the best operating conditions.

The two key parameters to consider during resin selection are binding capacity and selectivity. CEX can offer high binding capacities for monoclonal antibodies (up to 100 mg/mL) (30). Typically, static binding capacity can be measured in a high-throughput format for multiple loading conditions (pH and conductivity). These are indicative of the dynamic binding capacity trends that can then be measured for selected conditions. Intuitively, one would expect maximum CEX capacity to be obtained under lower conductivity and pH conditions since antibodies become increasingly protonated under acidic conditions. However, recent studies have shown that binding capacity for antibodies on CEX resins can increase unexpectedly with increasing conductivity and decreasing protein charge at the lower range of ionic strength conditions (34). This nontraditional behavior can be explained by an exclusion mechanism whereby the antibody can bind to the outer pore region and can electrostatically hinder subsequent antibody molecules from entering. Increasing the ionic strength and pH shields the charges on the protein and dampens the exclusion effect, thereby resulting in a higher capacity. As the pH and conductivity increase, the traditional trend of decreased capacity reasserts itself due to reduced interactions between the resin and the antibody. This

TABLE 7.2 Commonly Used CEX Resins

Resin Name	Vendor	Ligand	Base Matrix	Average Particle Size, $\mu\text{m}$	Pore Size, $\text{\AA}$
SP 650 S	Tosoh	Sulfopropyl	Methacrylate	35	1000
SP 650 M				65	
SP 650 C				100	
GigaCap S-650M	GE Healthcare	Sulfo	6% cross-linked agarose	75	n/a
SP Sepharose FF		Sulfopropyl	Highly cross-linked agarose with dextran surface extender	90	
Captio S			6% cross-linked agarose with dextran surface extender		
SP Sepharose XL			6% cross-linked agarose with dextran surface extender		n/a
Fractogel EMD SO3	Merck KGaA	Sulfoisobutyl Sulfoethyl	Methacrylate resin with polymeric "tentacles"	65	800
Fractogel EMD SE (M), Hicap					
Unosphere S	Bio-Rad	Sulfo	Polymeric	120	700–2000
S Ceramic Hyper D	Pall	Sulfo	Ceramic bead filled with a hydrogel	50	n/a
POROS HS50	Applied Biosystems	Sulfopropyl	Coated cross-linked poly(styrenevinylbenzene)	50	1600



**FIGURE 7.1** Selectivity plot for HMW removal. Linear gradient experiments were carried out with moderate loading and NaCl salt gradient at specified pH.

observation is important to keep in mind during process development while arriving at the optimal conditions for binding capacity.

Linear gradient experiments over a range of mobile phase pH and buffers can be conducted to screen resin selectivity. A plot of cumulative key impurity level vs. cumulative product yield provides an effective means to compare the selectivity of various resins without differences in peak shape and retention biasing the comparison (32) (Fig. 7.1). Batch screening carried out in high-throughput mode can also facilitate the screening of a large number of conditions, and such platforms have become commercially available. Figure 7.1 shows that the intrinsic selectivity for HMW aggregate removal is greater at lower pH values for both resins. Resin 1 outperforms resin 2 at pH 6.0 and 7.0 but is comparable at pH 5.0. Similar comparisons can be carried out for other impurities to provide guidance on resin selection and operating conditions, to maximize product yield and purity. Although selectivity is tested in linear gradient mode, process operations typically operate in a step gradient mode for manufacturing simplicity and ease of control. Step gradient conditions can be developed based on linear gradient data and some further optimization for yield and impurity removal.

## 7.2.2 HIC

HIC is based on the interaction between hydrophobic ligands (aliphatic or aromatic) and hydrophobic residues on the protein surface. This mode has

been used for protein purification since 1973 (35, 36) and has gained popularity over the years (see Chapter 5). In comparison with reversed-phase chromatography (RPC), HIC employs milder separation conditions that help to minimize protein denaturation. In HIC, protein adsorption on the stationary phase increases with salt concentration and elution is achieved by decreasing salt concentration (37, 38). HIC and IEX separations are based on completely different mechanisms and are often used as orthogonal polishing steps for protein purification.

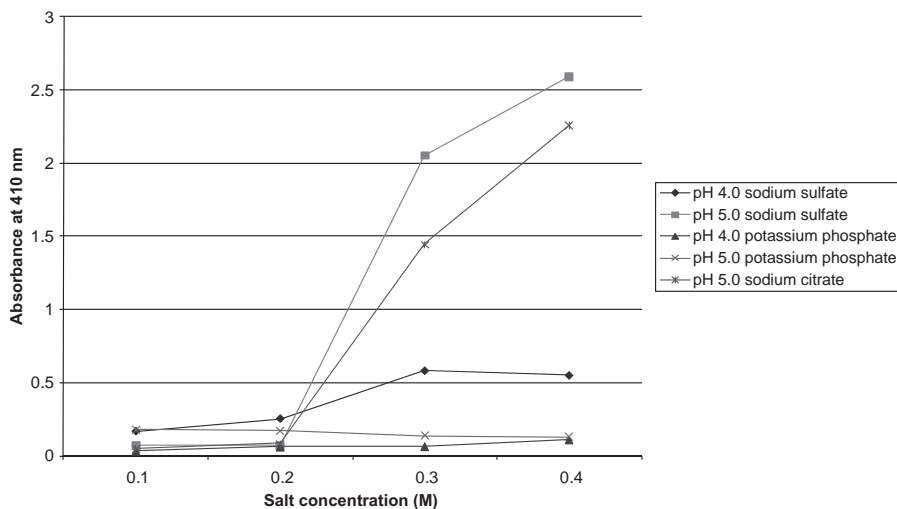
While HIC is not the most common mode of chromatography in antibody purification, it is used as a polishing step when HMW aggregate and HCP removal are primary concerns. Since HIC has an orthogonal mechanism to IEX, it offers distinct selectivity for removing HCP species that are not cleared by that mode of chromatography. HIC is usually very effective for the reduction of HMW aggregates as the latter are generally more hydrophobic and are better retained on HIC resins. HIC is inferior to IEX for the removal of leached Protein A and DNA, the latter tending not to bind under the high-salt conditions used in HIC. HIC can be operated in either flow-through or bind-and-elute modes depending on the impurities to be removed. HIC resins usually offer relatively low loading capacities in the bind-and-elute mode, so flow-through mode is preferred.

Table 7.3 lists the commonly available HIC resins that can be used in antibody purification. The choice of the resin is based on resin characteristics, selectivity for impurity removal and binding capacity. A recent product introduced specifically for antibody purification is the 600 series from Tosoh, in which the pore size has been optimized to maximize binding capacity and recovery.

A series of experiments is needed to screen for the best HIC resin and to develop optimal operating conditions. These typically include the generation of salt precipitation profiles, linear gradient retention studies, linear gradient selectivity studies, and an evaluation of dynamic binding capacities (39). A salt precipitation curve for the antibody of interest should be prepared in the presence of various lyotropic salts to identify the highest salt concentration that can be tolerated. This is important because higher salt concentrations often translate to higher binding capacities, but this can also lead to antibody precipitation. The salt precipitation curves help to identify a safe operating regime and narrow down loading salt conditions that need to be tested in subsequent development work. Protein precipitation can be evaluated by measuring the turbidity of the solution through light scattering or absorbance measurements at 410 or 600 nm. A precipitation profile is shown in Fig. 7.2. Since precipitation is a function of protein concentration, contact time, pH, temperature, salt type, and salt concentration, such profiles can be generated for each condition if needed. Along with turbidity measurements, SEC analysis of the samples is recommended since the salt and pH conditions that promote the formation of soluble aggregates can be different from those causing precipitation.

**TABLE 7.3 Commonly Used HIC Resins**

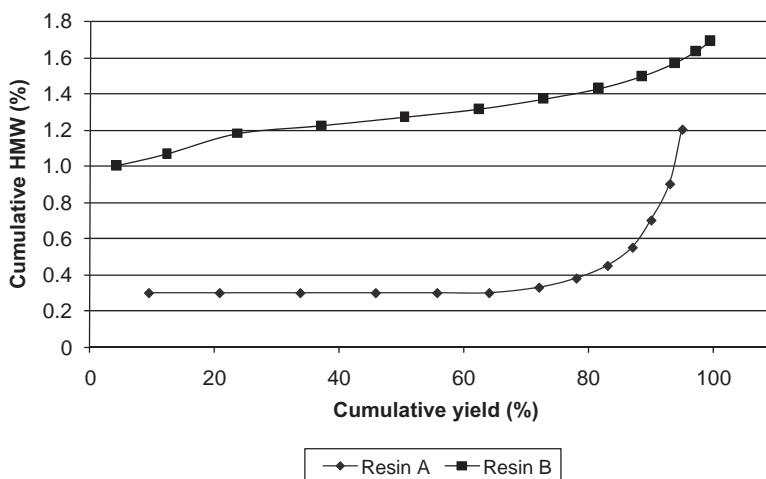
Resin Name	Vendor	Average Particle Size, $\mu\text{m}$	Ligand	Base Matrix	Pore Size, $\text{\AA}$
Butyl Sepharose HP	GE Healthcare	34	n-Butyl	Cross-linked agarose	n/a
Phenyl Sepharose HP			Phenyl		n/a
Butyl-S Sepharose 6 Fast Flow		90	Butyl-S	6% cross-linked agarose	n/a
Butyl Sepharose 4 Fast Flow			n-Butyl	4% cross-linked agarose	n/a
Phenyl Sepharose 6 Fast Flow (low sub)			Phenyl	6% cross-linked agarose	n/a
Phenyl Sepharose 6 Fast Flow (high sub)			High sub has higher ligand density than low sub		n/a
Octyl Sepharose 4 Fast Flow	Tosoh Bioscience	100, C	n-Octyl	4% cross-linked agarose	n/a
Toyopearl Ether-650			n-Butyl		1000
Toyopearl PPG-600M			Polypropylene glycol		750
Toyopearl Phenyl-650		35, S	Phenyl	Methacrylate	1000
Toyopearl Butyl-650			n-Butyl		1000
Toyopearl Butyl-600M			n-Butyl		750
Toyopearl Phenyl-600M			Phenyl		750
Toyopearl Hexyl-650			n-Hexyl		1000



**FIGURE 7.2** A precipitation profile with salt concentration vs. antibody absorbance (410 nm) after 1 h, antibody concentration of 4 mg/mL.

Once the safe operating regime for salt concentrations is defined, linear retention experiments can be carried out under decreasing salt gradients on various HIC resins to estimate the affinity of the protein at different salt and pH combinations. These experiments can help assess the hydrophobicity of the molecule and can guide resin selection accordingly. Resins that cause the antibody to elute too early in the gradient should be avoided in bind-and-elute mode as very high salt concentrations would be required for optimal binding. On the other hand, very hydrophobic resins that cause peak splitting or low recovery should also be avoided as they can denature the product. Following the retention study, linear gradients can be run under preparative loading conditions to generate selectivity plots as described in the previous section. In contrast to the retention study, the selectivity studies compare the resolution of different resins (for the impurity of interest) under a variety of mobile phase conditions. A selectivity plot comparing the ability of two resins to remove HMW aggregates is shown in Fig. 7.3. The shape of the curves indicates that resin B is more selective than resin A.

The methodology adopted for HIC development can be slightly different according to whether the bind-and-elute or flow-through mode is used. For bind-and-elute mode, the selectivity plot allows the developer to choose appropriate loading conditions to maximize capacity without product denaturation or aggregation, appropriate wash conditions if the impurity is retained, and appropriate elution conditions to maximize product recovery. For flow-through mode, weak binding resins can be considered. However, the use of a more hydrophobic resin with salt conditions that favor weak or no retention can often provide greater selectivity. Developing the load salt concentration



**FIGURE 7.3** A selectivity plot comparing the ability of two HIC resins to remove HMW aggregates, with sodium citrate buffer pH 4.8.

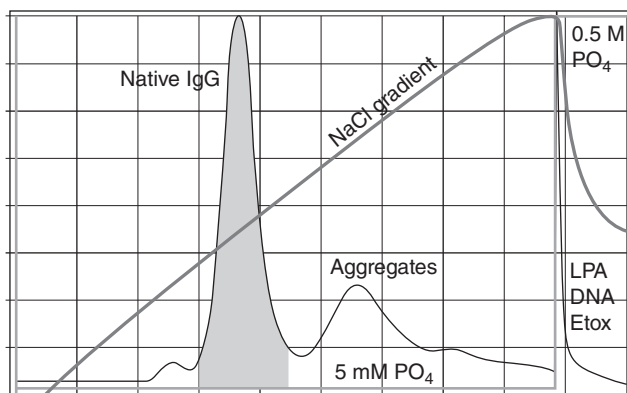
that provides best trade-off between product recovery and impurity elimination is very important for flow-through steps.

### 7.2.3 HA Chromatography

HA is a calcium mineral with the formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . In contrast to other adsorbents used in downstream processing, HA is both the ligand and the supporting matrix (40). There are two types of binding sites on HA—the positively charged calcium referred to as the C site and the negatively charged phosphate referred to as the P site. Depending on the pI of the product and the operational pH, electrostatic interactions with these sites can be either cationic or anionic. These ionic interactions can be disrupted by increasing the salt concentration. Carboxyl groups on the protein surface can also interact with the calcium sites by metal–chelate interaction. The binding is an order of magnitude stronger than normal IEX and increased salt concentration does not disrupt such interactions. Phosphate ions have proven effective for disrupting both metal–chelate and ionic interactions resulting in dissociation (41–43). The relative contributions of these interactions are case specific and depend on the surface properties of the protein.

HA chromatography is a very selective polishing step in antibody manufacture and is able to remove HCP, aggregates, and leached Protein A (44). Aggregates and Protein A–IgG complexes tend to be better retained than most antibodies in this mode. In addition, DNA usually elutes after most antibodies due to the strong interaction between the calcium sites and the DNA phosphate backbone. Typical operating conditions involve eluting the





**FIGURE 7.4** Distribution of impurities under typical operating conditions when eluting an antibody in a NaCl gradient with a small amount of additional phosphate (43). LPA: leached Protein A. Etox: Endotoxin.

antibody in a NaCl gradient with a small amount of additional phosphate (44). The distribution of impurities in such a gradient is shown in Fig. 7.4.

HA resins are available from two vendors: (i) a spherical, macroporous form of HA called ceramic HA (CHT Types I and II) from Bio-Rad (Hercules, CA, USA) and (ii) an HA agarose composite called Biosepra HA Ultrogel sorbent available from Pall (St. Petersburg, FL, USA). The former is more frequently employed in industrial applications because it is noncompressible and thus has superior pressure–flow properties. The large-scale packing of HA can be challenging and special precautions need to be taken to prevent rapid settling (which can lead to bed heterogeneity) and particle fracturing.

## 7.2.4 Mixed-Mode and Other Modes of Chromatography

Mixed-mode chromatography, as the name indicates, exploits a combination of interaction mechanisms to enable separation (see Chapter 6). These interactions can be simultaneous IEX and hydrophobic interactions, CEX and AEX, or IEX with biorecognition. The interplay of multiple interaction mechanisms can provide unique selectivity under conditions more suitable for manufacturing process flow.

Early research has shown that protein retention and resolution can be increased by incorporating more hydrophobic moieties onto AEX resins (45). The development and launch of mixed-mode resins has significantly accelerated in recent years, driven by a need to achieve greater selectivity in a single processing step. Based on initial high-throughput screening and prototype evaluation (46, 47), two commercial multimodal resins called Capto adhere and Capto MMC have recently been introduced by GE Healthcare. These combine AEX and CEX with hydrophobic moieties on an agarose backbone.

These resins enable IEX chromatography loading under high-salt conditions, which can simplify process flow by eliminating dilution or diafiltration requirements. These multimodal resins can also offer unique selectivity for the removal of impurities such as HMW aggregates or leached Protein A compared to traditional IEX. The process development methodology for mixed-mode resins is usually similar to their AEX and CEX counterparts if used under conditions where the IEX mechanism dominates. It is noteworthy that when operating these multimodal resins in bind-and-elute mode, elevated salt concentrations may not always be an effective strategy for protein elution due to secondary hydrophobic interactions. It has been shown that modifying the pH or adding chaotropic reagents can be necessary to facilitate complete recovery (48). Capto adhere should be used in flow-through mode much like traditional AEX resins for typical antibodies (GE Healthcare application note 28-9078-89 AA), with pH, salt concentration, and loading as the main operating variables that need to be optimized to achieve the best trade-off between yield and purity. The vendors note that the use of these mixed-mode resins can result in a two-step antibody purification process (GE Healthcare application note 28-9078-92 AA). However, more comprehensive evaluations need to be conducted for this strategy to find wider acceptance.

Another type of mixed-mode chromatography introduced for antibody purification is hydrophobic charge induction chromatography (HCIC). This employs a heterocyclic ligand such as 4-mercaptoethyl pyridine (MEP), which becomes positively charged at low pH values (49). While adsorption at neutral pH is based on hydrophobic interactions, elution is facilitated at low pH by charge repulsion between the ionized ligand and charged residues on the protein. Initial HCIC studies have focused on its use as a capture step in antibody processing (50, 51). However, recent studies have revealed its limited selectivity as a capture step compared to Protein A (52). HCIC might have more potential as a polishing step in place of traditional HIC since it operates under low salt conditions (53). Moreover, the combination of hydrophobic interactions along with electrostatic repulsion could also provide unique selectivity for impurity removal compared to traditional HIC matrices. More recently, two new resins based on hexylamine (HEA) and phenylpropylamine (PPA) ligands have also been introduced. Binding on these resins occurs through a combination of hydrophobic interactions (aliphatic hexyl group for HEA or aromatic phenyl group for PPA) and electrostatic interactions (through the amine group), while desorption is facilitated through electrostatic repulsion as described above (54). There is as yet no comprehensive understanding of how these two resins perform in mAb purification. Table 7.4 summarizes some of the mixed-mode resins that are commercially available and that can be employed as a polishing step in antibody manufacturing.

Recently, dye-ligand affinity chromatography has been used for the purification of a mAb from cell culture (55). Cibacron Blue resin contains a synthetic polycyclic dye ligand that can bind to a variety of proteins by hydrogen bonding, van der Waals forces, hydrophobic and ionic interaction, and in some

**TABLE 7.4 Commercially Available Mixed-Mode Resins**

Resin Name	Vendor	Ligand	Base Matrix	Average Particle Size, $\mu\text{m}$	Interactions
Capto MMC	GE Healthcare	Multimodal ligand containing carboxylic and phenyl group	Highly cross-linked agarose	75	CEX and hydrophobic interaction
Capto adhere		N-benzyl-N-Methyl ethanolamine	Highly cross-linked agarose		AEX and hydrophobic interaction
HEA HyperCel	Pall	n-Hexylamine	Cross-linked cellulose	80–100	AEX and hydrophobic interaction
PPA HyperCel		PPA			AEX and hydrophobic interaction
MEP HyperCel		4-Mercapto-ethylpyridine			Hydrophobic charge induction
Blue Trisacryl M		Cibacron Blue F3GA	Polymeric	40–80	Electrostatic and hydrophobic interaction

cases bioaffinity (the ligand is a NAD cofactor mimic) (56, 57). It has been used in earlier commercial processes for the production of albumin and thyroid-stimulating hormone, but only recently has it been evaluated as a potential mAb polishing step. This resin has been used as the second polishing step (in flow-through mode) following the capture for an IgG4 by Protein A chromatography. The resin has a high loading capacity and has significantly reduced the levels of bovine serum albumin (BSA), HCP, HMW aggregates, and degradation products. Several Protein A mimetic ligands have also been developed based on triazine dye chemistry (58, 59). While their performance as the antibody capture step has not been very promising (52), the selectivity afforded by their unique chemistries could be employed as a polishing step.

### 7.2.5 Dedicated Virus Removal Steps

In addition to virus clearance achieved by polishing, the ICH Q5A guidance document requires two dedicated virus clearance steps with orthogonal mechanisms (see Chapter 8). The two most frequently used processes are low-pH virus inactivation and filtration. The low-pH inactivation step is typically

placed after the Protein A step since the Protein A elution pool is already at a relatively low pH. Monoclonal antibodies tend to endure exposure to low pH conditions with no ill effects. However, if the antibody is unstable at low pH, other techniques such as solvent–detergent treatment may be used instead (60).

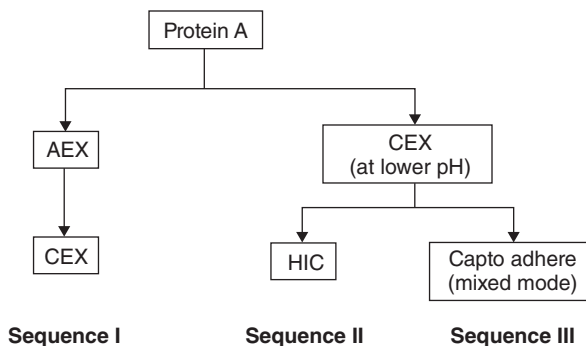
Filtration involves the size-based removal of virus particles by passage through a membrane with a small pore size. Filters are available in two categories based on their pore size ratings—retroviral (<50 nm) or parvoviral (<20 nm) (61). For more recent antibody products, the industry is favoring the use of parvoviral-grade filters to satisfy heightened stringency in regulatory expectations. Parvoviral-grade filters typically need a larger surface area because they tend to clog even in the presence of low levels of particulates and aggregates in process streams. As a result, virus filtration can be the second most expensive step in the process after Protein A chromatography, and the optimization of this process step and its integration into the overall purification sequence are equally important. The virus filtration step is typically placed after at least one of the polishing chromatography steps. The choice is usually based on product stream volume considerations as well as the volume that can be feasibly filtered for that process intermediate (62).

### 7.3 INTEGRATION OF POLISHING STEPS

The previous sections briefly described the nature of the polishing steps in antibody manufacture and provided several key considerations concerning process development. Additional considerations need to be kept in mind while integrating the above-mentioned polishing steps into a downstream process. One key factor is that of overall process productivity. Tugcu and colleagues (63) have recently described a two-stage resin screening approach that integrates chromatographic steps with a view toward maximizing productivity. Apart from productivity, other factors that influence the choice and number of polishing steps, their mode of operation, and their placement include (i) key impurities that need to be removed, (ii) how difficult they are to separate, (iii) final product purity constraints, (iv) optimal process flow with the fewest operational steps, and (v) how easy they are to fit in the manufacturing facility. Some of the philosophies concerning the integration of polishing steps into a manufacturing process are illustrated through the following two case studies.

#### 7.3.1 Case Study I: Selection and Placement of Polishing Steps

The following is a case study concerning the sequence of polishing steps chosen for a mAb purification process. Based on initial development, three process sequences were proposed for further evaluation as shown in Fig. 7.5. All sequences started with the same Protein A capture step and a low-pH hold for viral inactivation. Following that, Sequence I adjusted the Protein A elution



**FIGURE 7.5** Proposed process sequence choices for mAb purification.

pool to an alkaline pH, then used AEX in flow-through mode followed by CEX in bind-and-elute mode. Note that the AEX step was placed before the CEX step to facilitate process flow because the Protein A elution pool had a lower conductivity than the CEX elution pool, so placing AEX as the second step eliminated the requirement for load dilution or buffer exchange. Moreover, the AEX flow-through pool was alkaline and could be loaded directly onto the CEX column. However, adjusting the pH of the Protein A pool to the neutral range prior to the AEX step caused a large amount of impurities to precipitate. Although the precipitate could be removed by filtration, evaluation of the filtration process suggested that it posed a significant manufacturing challenge due to the inherent variability in the precipitation process and the large filter area required to achieve adequate clarification.

Sequences II and III both used CEX as the second step. This was operated in bind-and-elute mode at a lower pH to avoid the precipitation that occurred in Sequence I, at the same time improving the selectivity of the CEX step for HMW aggregate reduction. The third step in Sequence II was HIC in the flow-through mode, which provided additional HMW aggregate removal but had somewhat limited DNA clearance capability. A key consideration in this scheme was the load preparation for the HIC step. The elevation in conductivity required for the HIC load was achieved by mixing the CEX elution pool with a high-salt buffer. The resulting product pool volume expansion introduced additional constraints on tank volume capacities for storing the HIC load and the product pool after HIC.

The third step in Sequence III was mixed-mode Capto adhere chromatography operated in flow-through mode. As mentioned before, this resin has both AEX and hydrophobic properties, allowing the antibody to bind at high conductivities. As a result, the CEX elution pool could be pH adjusted and loaded directly onto the mixed-mode column without further dilution or buffer exchange steps. Development data also revealed good DNA, HCP, and leached Protein A clearance for this sequence (data not shown). This sequence was

found to fit well into an existing manufacturing facility with the flexibility to accommodate increased cell culture titers in the future.

The pros and cons of each sequence are summarized in Table 7.5. This was based on data concerning the clearance of impurities, facility-fit analysis, and an overall economic evaluation. A final decision on the appropriate process sequence needs to take into account multiple factors including the nature and level of the major impurities, manufacturing convenience, facility fit, and cost considerations. As a case in point, if the dominant consideration is HMW aggregate levels and if tank capacity is not the governing issue, Sequence II might be a better choice than Sequence III. However, if DNA clearance is a concern, then Sequence III would be a better choice and this would also circumvent the limitations on tank capacity. If the issues surrounding precipitation were not dominant, Sequence I could also be a good choice as it provides superior DNA clearance.

### **7.3.2 Case Study II: Selection of Operational Mode and Influence of the Previous Polishing Step**

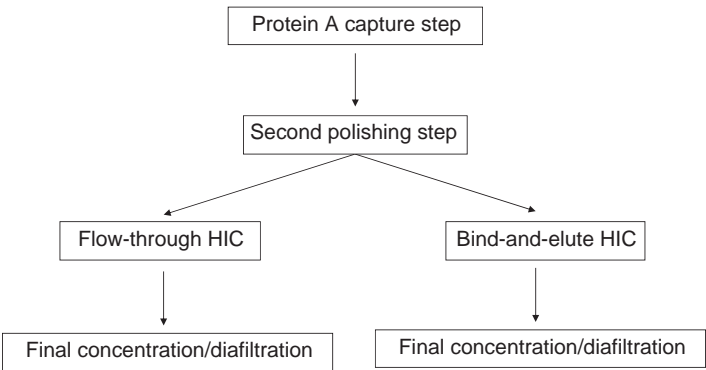
This case study shows the rationale for the selection of bind-and-elute mode versus flow-through mode for HIC in an antibody purification process. Figure 7.6 shows the two process sequences that were developed and Table 7.6 lists the operating parameters for the HIC steps. For this case study, the Protein A capture step and the first polishing step had removed the majority of the process-related impurities such as DNA, HCP, and leached Protein A. The main purpose for the HIC step was to reduce HMW aggregate levels. Both the modes were optimized to provide similar HMW aggregate reduction.

On comparing the operating parameters in Table 7.6, it can be seen that a significantly higher loading capacity was possible in flow-through mode, and the mobile phase salt concentration was also lower. This can have several operational advantages such as lower salt disposal costs and reduced tank corrosion. The lower salt concentration also enabled the use of a higher protein concentration in the load. The mAb concentration reached 8–10 g/L with no sign of precipitation in a mobile phase comprising 120 mM sodium citrate, whereas the highest mAb concentration achieved in the 500 mM citrate was ~2 g/L. The higher protein concentration translated into a lower load volume and a reduced loading time. This can be important from a facility-fit point of view if tank volumes in the load preparation area are the limiting factor. Under these circumstances, it would be desirable to operate the HIC step in flow-through mode.

On the other hand, flow-through also has some drawbacks and, in certain cases, the bind-and-elute mode can be more suitable. In an earlier version of the above-mentioned process, there was a specific HCP (referred to as impurity X) that was not removed by the previous polishing step. It was necessary to remove X for comparability purposes, and the subsequent HIC step had to

**TABLE 7.5    Analysis of Pros and Cons for Each Sequence Choice**

	Sequence I Protein A–AEX–CEX	Sequence II Protein A–CEX–HIC	Sequence III Protein A–CEX–mixed mode
Pros	Good DNA and HCP clearance  No load adjustment required for the AEX or CEX step	Selectivity of the CEX step for HMW was greater at lower pH Robust HMW clearance provided by the HIC step	Selectivity of the CEX step for HMW was greater at lower pH  Good HCP, DNA, and HMW clearance  Minimal load adjustment for all the steps
Cons	Precipitation can pose a significant filtration challenge  Limited HMW clearance capability	CEX elution pool had to be diluted for the subsequent HIC step  Limited DNA clearance capability for the HIC step	Mixed-mode resin is more expensive than traditional ion exchangers or HIC resins



**FIGURE 7.6    Process flow diagram for Case Study II.**

be optimized to remove both HMW aggregates and this specific HCP. X was less hydrophobic than the antibody and was therefore coeluted with the antibody in flow-through mode, making it an unfavorable option. However, for the bind-and-elute step, the salt concentration could be chosen carefully to elute X while the monomer and HMW aggregates bound to the resin during the loading step. Suitable wash conditions were also developed to wash impurity X through the column prior to elution. Then, during the elution step, the

**TABLE 7.6 Operating Parameters for the HIC Bind-and-Elute and Flow-Through Steps**

Operating Condition	Bind-and-Elute Mode	Flow-Through Mode
Loading capacity, mg antibody/mL resin	15–20	50–60
Loading salt concentration, mM citrate	500–600	100–120
Load protein concentration, mg/mL	<2	8–10

salt concentration was optimized to elute the antibody preferentially leaving the HMW aggregates bound to the resin.

For the case described above, the first polishing step was further optimized to remove impurity X during subsequent development. Thus, in the final process, the HIC step could be operated in the flow-through mode and could take advantage of the operational benefits mentioned in the previous paragraph. This also emphasizes the importance of looking into the downstream process as an integrated system rather than discrete chromatographic steps to achieve at the best combination of polishing steps.

## 7.4 CONCLUSIONS

It is not yet practical to use a single generic polishing step for antibody purification. The presence of the common Fc moiety in all antibodies allows for a generic capture step using Protein A chromatography, but the development of a polishing strategy is still case specific and needs to be tailored toward specific impurity removal requirements (which depend on the upstream process) and unique properties of each antibody, reflecting variable charge distribution and hydrophobic characteristics. Current best practice involves the use of two polishing steps following Protein A chromatography, but the industry is trying to move toward a single polishing step. Some of the chromatography strategies discussed in this chapter, such as AEX in weak-partitioning mode, the use of mixed-mode resins, or the use of membrane chromatography might help to achieve that vision. Several attempts have also been made to purify antibodies without a Protein A step (see Chapters 5 and 14). In such cases, the demand on the polishing steps would be even greater and significantly more method development time would be required.

## 7.5 ACKNOWLEDGMENTS

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## ORTHOGONAL VIRUS CLEARANCE APPLICATIONS IN MONOCLONAL ANTIBODY PRODUCTION

JOE X. ZHOU

### 8.1 INTRODUCTION

Recombinant monoclonal antibodies (mAbs) are important therapeutics for cancer and many other diseases (1–3) due to their long half-life and the feasible platform technologies for upstream and downstream processing (4). However, because of the way antibodies function, large doses are normally required for therapy, and this requires extremely high product throughput in each downstream process (4).

High throughput is generally achieved using Protein A (ProA) affinity chromatography, which is implemented on a large scale for commercial production. Over 98% of product purity is typically achieved with greater than 95% yield after ProA affinity chromatography (5–7). Polishing steps such as cation exchange (CEX), anion exchange (AEX), and hydrophobic interaction chromatography (HIC) are often used to remove trace amounts of process impurities including host cell protein (HCP), recombinant DNA (rDNA), leached recombinant ProA, as well as product-related impurities such as monoclonal aggregates (AGGs) and different charge variants (8). In large-scale antibody manufacture, these steps must be robust and must consistently deliver a high yield of extremely pure product (8). In addition, the process must demonstrate excellent clearance of all the chemical reagents from the cell culture medium, such as methotrexate (MXT), antifoam, and buffer components that should not be present in the final bulk formulation.

Thus far, the majority of mAbs have been produced in mammalian cells. These provide the glycosylation machinery to add the correct sugar residues onto mAb molecules to achieve a longer molecular half-life (9). When protein therapeutics are produced in animal cell lines, the risk of contamination with viruses from the cell line or with constituents of the culture media is a critical consideration (10). Therefore, to further ensure the safety of the product, multistep purification processes must be designed not only to remove product-related and process impurities, but also for the removal and/or inactivation of viruses (11–14). The large-scale production of mAbs thus continues to be a challenge (4, 8).

In order to satisfy the safety requirements of patients and to comply with regulatory guidelines while delivering an extremely high product throughput, several disposable systems have been tested and implemented in the large-scale production of therapeutic mAbs (10) (see Chapters 14 and 16). This is because such systems provide high product throughput, cost efficiency, and user-friendliness without cleaning, lifetime, and storage validation requirements (10). Recent presentations indicate that depth filtration, one of the most widely deployed disposable systems, is able to remove not only turbidity, HCP, and rDNA, but also different viruses (10, 15, 16). Additionally, published data demonstrate that Q membranes can efficiently remove different model viruses at high flow rates under a broad range of operational pressures (8). The viral clearance capacity of such membranes can exceed 3000 g/m<sup>2</sup> (17). Disposable systems are therefore becoming routine operations in mAb downstream processing.

In this chapter, the basic concepts of viral clearance and validation in mammalian cell expression systems are introduced. The performance of traditional chromatographic steps and disposable systems for viral clearance is reviewed, specifically with regard to the Chinese hamster ovary (CHO) cell system, and working mechanisms are discussed. The pros and cons of implementation in large-scale mAb purification are also explored.

## 8.2 MODEL VIRUSES AND VIRUS ASSAYS

Model viruses and assays for viral clearance studies have been extensively discussed in the key regulatory guidance documents (18–21). The summary below highlights some of the major points made in those documents, and the reader is referred to the references for additional details.

The selection of viruses for a virus clearance study depends greatly upon the nature of the starting materials and the reagents used to manufacture the product. Relevant viruses, defined as those viruses known to contaminate or likely to contaminate the starting materials and reagents, are used for clearance studies whenever possible. When a relevant virus or an appropriate assay

**TABLE 8.1 Virus Panel for a Rodent Cell Line-Derived Product**

Virus	Genome	Envelope	Family	Size, nm	Resistance Level
MMV	DNA	No	Parvo	20–25	High
PRV	DNA	Yes	Herpes	150–250	Medium
Reo-3	RNA	No	Reo	60–80	High
X-MuLV	RNA	Yes	Retro	80–110	Low

system for its detection and quantitation is not available, a model virus may be used as a substitute. Model viruses should be closely related to the relevant virus and should share physical and chemical properties with the relevant virus. For example, cell lines from rodents are known to contain endogenous retroviral particles or retroviral-like particles that may be noninfectious or infectious depending upon the cell line. The capacity of the manufacturing process to clear (remove and/or inactivate) rodent retroviruses from products derived from these cell lines should be determined. This may be accomplished using a murine leukemia model virus.

Although it is not possible to predict all the potential relevant viral contaminants for a particular process, the use of a panel of relevant and model viruses with a wide range of properties (including size, chemistry, and resistance to inactivation) will help to determine the ability of the purification process to clear both predicted and unknown viral contaminants. In other words, the ideal panel of viruses for a viral clearance study will show a broad diversity of physical and chemical characteristics such that the panel will challenge the robustness of the manufacturing process to clear any virus. Table 8.1 lists a panel of viruses recommended for the study of a product derived from a rodent cell line. The panel is composed of relevant and model viruses representing four different viral families and showing a wide range of physical and chemical properties. If all four of these viruses were adequately cleared by the manufacturing process, the manufacturer would have a high level of assurance that the process would be able to clear any virus—even the most unexpected viral contaminant.

Viral clearance samples should be assayed in such a way as to determine virus titer. The two assay methods most frequently used are plaque (or focus) assays and cytopathic effect (CPE) or 50% tissue culture infectious dose (TCID<sub>50</sub>) assays. Plaque assays are quantitative in that each plaque (or focus) corresponds to a single infectious unit, i.e., the infectivity correlates directly with dose. TCID<sub>50</sub> assays are quantal assays in that wells are scored as positive or negative for the presence of infectious virus in serially diluted samples, and the dilution required to infect 50% of the culture wells is then calculated. TCID<sub>50</sub> assays offer the advantage of determining the titer of viruses that do not produce plaques, but which do cause a change in cellular morphology.



The plaque and TCID<sub>50</sub> assays both lend themselves to statistical evaluation and validation. Variation can arise within an assay system from a number of sources. Variability associated with assay reagents should be well controlled. For example, only well-characterized viral and cell banks should be used in these assays. All the other reagents used in the assay should also meet appropriate specifications. Interoperator, interday and intra-assay variation may also affect the titration results. Assay validation permits the quantitation of each of these parameters.

The sensitivity of the titration assay is directly related to the volume of sample material tested. At low virus concentrations, small sample volumes may or may not contain infectious virus particles, and there is a discrete probability that if only a fraction of the sample is tested, the test result will be negative due to the random distribution of viruses throughout the total sample. In such situations, increasing the sample volume will increase the assay sensitivity. If only a portion of the sample is tested and the test is negative, the amount of virus that would have to be present in the total sample in order to achieve a positive result with 95% confidence should be calculated and taken into consideration when calculating a reduction factor.

The virus reduction factor is the log<sub>10</sub> ratio of the input virus load and the output virus load. The 95% confidence limits of the log reduction factor should be determined. The overall reduction factor for a complete manufacturing process is the sum of the log reduction factors of the individual steps tested.

### **8.3 VIRUS CLEARANCE STRATEGIES FROM FIRST IN HUMAN (FIH) TO BIOLOGICAL LICENSE APPLICATION (BLA) FILING**

The virus clearances strategies used in FIH or phase I processes are different from the strategies required in phase III and BLA or commercial process filing. FIH processes generally require the testing of two model viruses, such as X murine leukemia virus (MuLV) and mice minute virus (MMV) particles. The virus samples are assayed only once with a new resin. In this way, cost is controlled while the efficacy of the therapeutic mAb is evaluated in later-stage trials. Virus clearance also requires transmission electron microscopy (TEM) for the evaluation and the tabulation of retroviral-like particles (22).

The virus clearance studies and documentation for phase III and BLA filing prior to commercial launch is extremely comprehensive. A minimum of four model viruses, MMV, X-MuLV, pseudorabies virus (PRV), and reovirus 3 (Reo-3), is required to validate phase III and future manufacturing processes for most biotechnology-derived therapeutics. New and used resins are examined during BLA viral clearance validation in replicate assays. Three different TEM samples from different cell culture lots are assayed. Regulatory agencies are currently updating guidelines for viral clearance in mAb production, which may result in further requirements for industry to address.



## 8.4 ORTHOGONAL VIRAL CLEARANCE IN mAb PRODUCTION

### 8.4.1 Major Viral Clearance Steps: Capture, Low-pH Viral Inactivation, and Polishing

ProA capture chromatography is a strong virus removal step. As shown in Table 8.2, up to 2.8 log reduction can be achieved for MMV, up to 2.6 for Reo-3, up to 3.1 for PRV, and 1.3–4.2 for X-MuLV for this single unit operation (23). Additional polishing chromatography methods such as AEX, CEX, and HIC, summarized in Table 8.2, have also proven useful as viral clearance steps. HIC chromatography appears to be less effective than AEX or CEX, but more effective than ceramic hydroxyapatite (CHT) for the removal of all four model viruses (Table 8.2). AEX has been proven to be the most robust step for Reo-3, MMV, PRV, and X-MuLV, and a log reduction greater than 5 is achievable for all the viruses if operational conditions are optimized (24). Low-pH treatment for viral inactivation is more effective for enveloped viruses and has less impact or even no effect on nonenveloped viruses. Our data indicate that a greater than 3.1 log reduction is achievable for PRV and that 2.9–6.2 log reduction is achievable for X-MuLV particles (24).

### 8.4.2 Disposable Systems

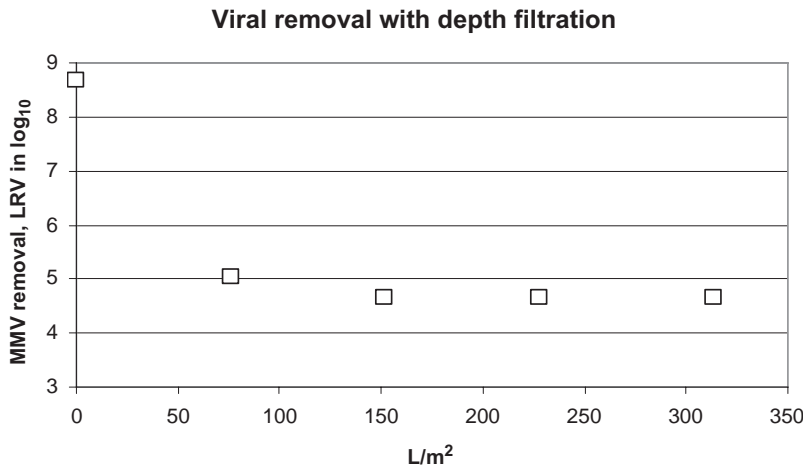
Newly developed disposable systems appear to be powerful virus clearance technologies (16, 23). The virus clearance capacity of depth filtration, Q membrane chromatography, and nanometer filtration are discussed below.

**8.4.2.1 Depth Filtration.** Our recent published data show that an A1HC depth filter was able to remove nonenveloped RNA viruses (such as MMV) ranging in size from 18 to 26  $\mu\text{m}$  at a process capacity of 320 L/m<sup>2</sup> when a feed containing 2% spiked viruses was loaded at 216 L/m/h (Fig. 8.1) (16). A log reduction value (LRV) of >4 was obtained over the entire course. The result was confirmed in our recent good laboratory practice (GLP) and non-GLP viral clearance studies (Table 8.3). In addition to the removal of MMV, the data listed in Table 8.3 also demonstrate that the filter can remove other model viruses including X-MuLV, PRV, and Reo-3 particles (16).

**TABLE 8.2 Virus Clearance Power Using Different Chromatography Steps**

Resin	Spike, %	Reo-3 (LRV)	PRV (LRV)	MMV (LRV)	X-MuLV (LRV)
CEX	5	2.6 → 6.2	>4.5 → 5.5	1.0–4.3	1.3 → 5.7
AEX	5	5.4 → 6.4	>3.9 → 6.7	4.5 → 6.8	>4.2 → 6.1
HIC	5	2.0–3.6	1.5 → 4.1	<1.0–2.4	<1.0 → 5
HA/CHT	5	<1.0	<1.0	1.8–2.1	1.6

HA: hydroxyapatite.



**FIGURE 8.1** Kinetic removal of MMV with depth filter.

**TABLE 8.3** Summary of Virus Reduction Using A1HC at pH 5.0 and 4mS/cm

Model Virus	First Experiment (LRV)	Second Experiment (LRV)	Process Capacity, L/m <sup>2</sup>
MMV	2.91 ± 0.30	4.13 ± 0.55	395
X-MuLV	2.57 ± 0.26	≥4.23 ± 0.36	400
PRV	≥3.69 ± 0.17	3.17 ± 0.64	400
Reo-3	≥3.97 ± 0.26	≥5.01 ± 0.35	393

This finding (16) is in agreement with a report discussing the virus removal ability of CUNO VR05 and VR07, whose mechanism is charge based (25). The stronger-charged membrane (VR07) achieved a higher LRV (1.4–2.0 for VR07 and 0.9–1.1 for VR05) when the model parvovirus PPV was tested (25). The slight difference can be ignored since the assay standard deviation (SD) is  $\pm 1$  LRV. When depth filters (with either a strong or a weak charge) were challenged with X-MuLV particles, a  $>4.8$  LRV was obtained with both filters. The operation was carried out at pH 5.0 with a low conductivity (20mM sodium acetate). The pI of PPV is approximately 5 (26, 27) and that of X-MuLV is approximately 6.7 (28). It is logical that charge-based membranes should perform better on particles with higher pI values than the operational pH and worse when the operational pH is similar to the viral pI value.

Our experiment was performed under almost the same conditions (16). At pH 5.0 and at a conductivity of less than 4 mS/cm, an LRV of  $>3$  was obtained for all virus particles. It seems that the different clearance mechanisms involved are hydrophobic compared to charge-based chemistry. In order to confirm this, the depth filtration was performed at a conductivity of 13 mS/cm and at pH

**TABLE 8.4 Summary of Virus Reduction at pH 7.0 and 13mS/cm**

	Capacity, L/m <sup>2</sup>	X-MuLV (LRV)	PRV (LRV)	MMV (LRV)
A1HC	200	4.69	3.58	1.66
CUNO 90ZA	200	4.14	2.97	0.8

**TABLE 8.5 Virus Clearance Power of Q Membrane Chromatography**

Run	Process Capacity, kg/m <sup>2</sup>	Flow Rate, cm/h	X-MuLV (LRV)	PRV (LRV)	Reo-3 (LRV)	MMV (LRV)
A	3.0	450	≥5.35	≥5.58	≥7.00	≥6.03
B	3.0	450	≥5.52	≥5.58	≥6.94	≥6.03
C	3.6	600	≥5.59			

7.0 to minimize the charge effects. The results in Table 8.4 show that under these experimental conditions, virus particles with a pI closer to operational pH were cleared better by the filters. In comparison, since MMV has a pI of 5, far from the operational pH, only minimal clearance of MMV was achieved by both filters. The high conductivity solution seemed to improve the clearance of X-MuLV and PRV particles, which had pI values ranging from 7.4 to 7.8 (29). The results clearly demonstrate that viral clearance by the depth filters used in that study involved hydrophobic mechanisms. Since the second study was performed using cell culture media containing rich nutrients such as peptone and yeast extracts, and these protein-based nutrients might compete in a hydrophobic base, a lower LRV could be expected for X-MuLV and PRV particles compared with when a pure chemical solution is used for the virus clearance study.

The performance consistency and integrity of these depth filters are critical considerations, although the data reported are the first to showcase their virus clearance power using four different models. These filters have been used consistently for reproducible HCP and rDNA removal at scales ranging from a few liters to 2000 L in clinical production. Therefore, establishing qualification methods for the depth filters based either on integrity or functionality will significantly increase the confidence levels for regulatory agencies and for future manufacturing implementation.

**8.4.2.2 Q Membrane Chromatography.** Q membrane chromatography has been tested and reported recently (8, 16, 30, 31). The results, as summarized in Table 8.5, demonstrate the virus clearance potential when four model viruses were tested at pH 7.2 and at approximately 4 mS/cm. A virus recovery of 100% was obtained in duplicate runs with a 1% virus spike except the runs using X-MuLV viruses, which achieved 70% virus recovery ( $n = 3$ ). The explanation for this lower value for X-MuLV may be that the strip solution, 1 M NaCl, inactivated some viruses that were irreversibly bound to the membrane or that

**TABLE 8.6 Virus Clearance Lot-to-Lot Variability Using Q Membrane**

Lot Number	A	B	C	Average	SD
LRV	6.6	6.73	6.73	6.69	0.08

**TABLE 8.7 Temperature Effects on MMV Clearance**

Temperature, °C	Process Capacity, kg/m <sup>2</sup>	Flow Rate, cm/h	Conductivity, mS/cm	pH	Pressure, psi	MMV (LRV)
8	3	450	4	7.2	29	≥6.03
13	3	450	4	7.2	22	≥6.03
20	3	450	4	7.2	13	≥6.12

**TABLE 8.8 Effect of Flow Rate on X-MuLV Removal**

Flow Rate, cm/h	Process Capacity, kg/m <sup>2</sup>	Conductivity, mS/cm	pH	X-MuLV (LRV)
450	3.0	4	7.2	≥5.35
450	3.0	4	7.2	≥5.52
600	3.6	4	7.2	≥5.59

were inactivated by mechanical stress during the process of adsorption and desorption.

When a Q membrane is used in flow-through mode at high flow rates, a major concern is the lot-to-lot variability of such small devices. Table 8.6 presents the LRVs achieved when three different lots of Sartobind Q 125 were used to remove PRV (16). It was found that the LRV obtained from each run is close to the others with a final average of 6.69 LRV and an SD of 0.08. These data were confirmed using MMV small particles (data not shown), and an SD of 0.18 was achieved. The data suggest that the Q membrane is a reliable device for virus clearance with minimal lot-to-lot variability despite using a high flow rate.

When the scale model of Sartobind Q 125 was tested, viral clearance was examined with MMV particles at different operational temperatures (31). The data summarized in Table 8.7 indicate that, although the operational pressure at 3 kg/m<sup>2</sup> reached 29 psi at 8°C, the LRV obtained from this run is comparable to that obtained at 13 psi and 20°C (room temperature) (31). This suggests that room temperature should be used to achieve the best process capacity without operational pressure limitation. The high operational pressure seems not to affect the virus clearance ability of Q membranes (31).

Using X-MuLV as a model virus, we also tested two different flux rates and a comparable LRV was obtained, as summarized in Table 8.8 (16). Using MMV

**TABLE 8.9 Maximum Virus Clearance Capacity of Q Membrane**

Process Capacity, kg/m <sup>2</sup>	Flow Rate, cm/h	Conductivity, mS/cm	pH	MMV (LRV)
3.0	450	4	7.2	≥6.03
3.0	450	4	7.2	≥6.03
5.04	450	4	7.2	≥6.31
6.31	450	4	7.2	≥6.97

**TABLE 8.10 Effects of Operational pH on MMV Removal at 4 mS/cm**

Process Capacity, g/m <sup>2</sup>	Operational pH	Virus Clearance (LRV)
200	6.4	4.77
200	7.22	5.53
200	7.99	5.96

**TABLE 8.11 Effects of Operational Conductivity on MMV Removal at pH 7.2**

Process Capacity, g/m <sup>2</sup>	Operational Conductivity, mS/cm	Virus Clearance (LRV)
3000	4	5.09
3000	5	4.47
2500	6	4.29

as a model virus, the maximum virus clearance capacity we have achieved so far is 6.3 kg/m<sup>2</sup> as shown in Table 8.9 (16).

Since the Q membrane is a charge-based device, changes in operational pH and conductivity will also affect the efficiency of virus clearance, and suitable pH and conductivity ranges will determine the robustness of virus clearance. Because no suitable scale-down model was available at the time, we used Sartobind Q 75, with very limited operational pressure, and as little as 200 g/m<sup>2</sup> of process capacity was achieved (16). Based on our previous study, we found that there was no negative impact on virus removal if the process capacity was limited by operational pressure. The effect of operational pH on MMV removal is summarized in Table 8.10. From the data, it is clear that the LRV value for MMV removal is higher when the operational pH is higher. This seems very reasonable since the virus particle has a pI of 5 and the experiment was performed at pH 6.4 or above, with the highest LRV achieved at pH 7.99.

The virus clearance data with MMV particles was later determined using 1 mL of the nano-Sartobind Q device (40 cm<sup>2</sup>), and the data are summarized in Table 8.11 (16). The data demonstrate that the virus clearance power was slightly reduced when the operational conductivity was increased. Since the nano-Sartobind Q is a newly developed scale-down model that better mimics the large-scale device, the 3000 g/m<sup>2</sup> capacity was readily achieved.

Thus far, our published data demonstrate that the Q membrane can remove all of the model viruses. The data seem to suggest that operational parameters such as pH, conductivity, flow rate, and temperature can be adjusted on a case-by-case basis to achieve the required LRV for different viruses. When feed containing different proteins is spiked with virus, varied LRV values may be obtained due to the macromolecular interactions. In addition, we found that when using 10 g/L of mAb for a lot-to-lot study, a consistent LRV was obtained from three different lots of membrane. However, the LRV value achieved at 10 g/L was only 60% of that achieved at 4.5 g/L (data not shown).

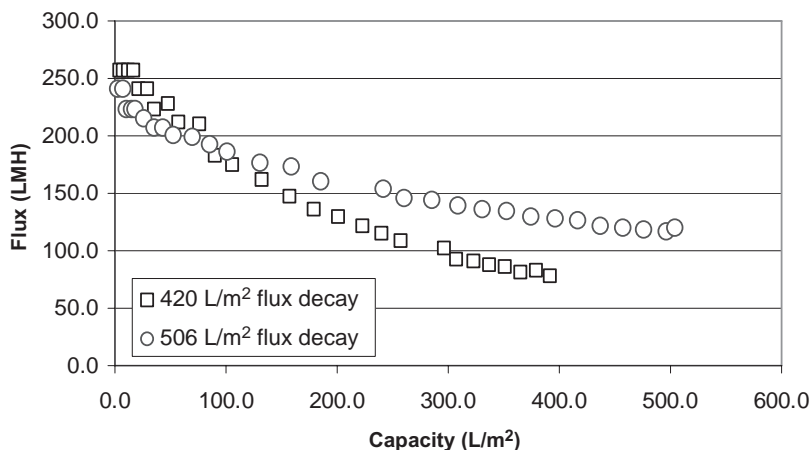
The sensitive detection of any defects in the Q membrane remains an issue, particularly when the device is used at a high flow rate with a minimal bed height in flow-through mode. Regular asymmetry and height equivalent to a theoretical plate (HETP) qualification can examine the separation power, but it is extremely difficult to detect microdefects in flow-through mode. The same concept applies to column operation: asymmetry and HETP cannot detect microchanneling in flow-through mode when using a defined column. However, it is safer when the column is operated at relatively slow flow rates since its bed height is much larger than that of a Q membrane device. Although the current pressure or bubble testing can be used for Q membrane integrity qualification and model proteins can be used to determine binding capacity, more sensitive detection of defects is desirable, particularly for future large-scale manufacturing (16).

**8.4.2.3 Twenty-Nanometer Filtration.** Sizing-based 20-nm filtration is a robust step for the removal of most viral contamination, and it is commonly used in almost all therapeutic protein production processes based on mammalian cells. Table 8.12 summarizes our recent LRVs achieved for four model viruses using a normal flow parvovirus (NFP) filter (16). Similar results were achieved for each of the model viruses when two different process capacities (420 and 506 L/m<sup>2</sup>) were compared (16).

Different lots of membrane were used in this virus clearance study and, although the flux decay in the two runs for the same model virus was different, the LRVs were similar. For instance, the same LRV was obtained for X-MuLV clearance in each of the runs. At a process capacity of 420 L/m<sup>2</sup>, 79% flux decay

**TABLE 8.12 Virus Removal with NFP 20-nm Filtration**

Process Capacity, L/m <sup>2</sup>	MMV Clearance and Flux Decay		Reo-3 Clearance and Flux Decay		X-MuLV Clearance and Flux Decay		PRV Clearance and Flux Decay	
	LRV	%	LRV	%	LRV	%	LRV	%
420	>5.92	49.3	>4.52	56	≥4.87	79	>4.43	47
506	5.92	26.1	≥5.22	35	≥4.87	54	≥5.04	60



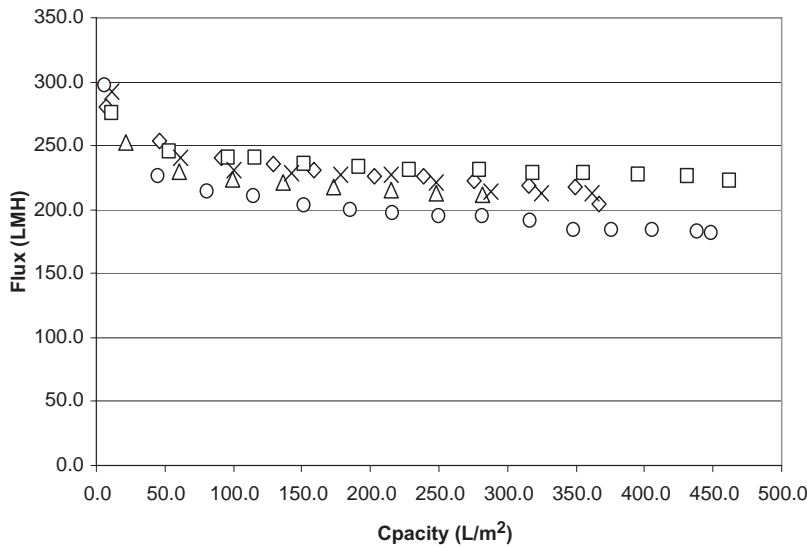
**FIGURE 8.2** Flux decays of X-MuLV virus filtration at process capacities of 420 and 506 L/m<sup>2</sup>.

was observed. In contrast, at a process capacity of 506 L/m<sup>2</sup>, the flux decay was reduced to 54% (Fig. 8.2). This may indicate lot-to-lot variability in both membrane and virus preparation, although virus removal power was not affected.

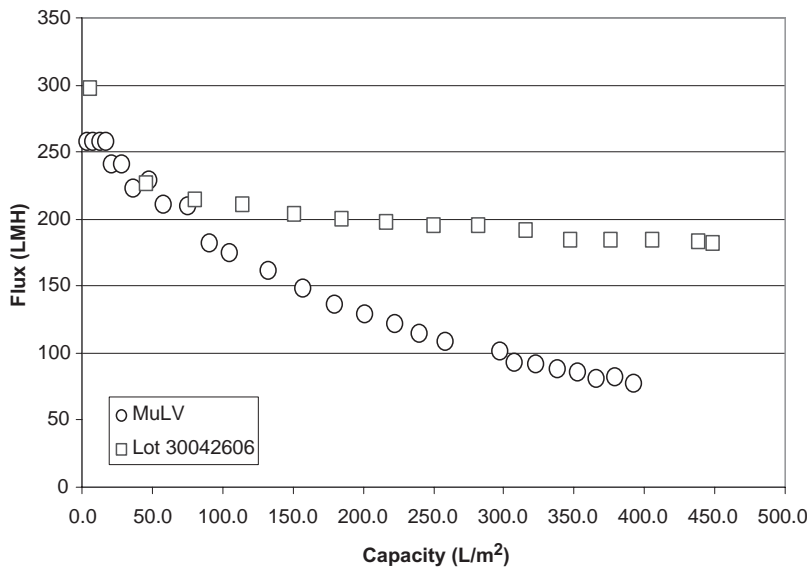
The model viruses are only used for process step validation with scale-down models. Model viruses are not used to spike the actual manufacturing process, so the observed flux decay is different from that found in a virus clearance validation study. Figure 8.3 shows the flux decay over five runs at the 2000:1 scale in a pilot plant, using a 10-inch (25-cm) device. The flux decay ranged from 19.2% to 38.9% with the feed materials making a major contribution. This indicates that the difference in flux decay between the viral spike study and the pilot plant runs presented in Fig. 8.4 predominantly reflects the virus particles and media components. Based on these data, oversizing the 20-nm filter surface in practice is a major concern.

When different viral preparations were examined using the same filter membrane and feed materials, it was found that the virus clearance capacity was different (Fig. 8.5) (16). Since the viral stock had a lower titer, a 0.2% spike was used from viral clearance contract laboratory B (Vendor B). The LRV values summarized in Table 8.13 indicate that indeed some viral preparations generated a higher flux decay, which contributed to the low process capacity of filterability (Vendors A and B in Table 8.13 and in Fig. 8.5), although their viral clearance abilities appear similar, and both are greater than 3.17 LRV in a large-volume assay.

When the MMV particle was used for the virus filtration study, poor virus preparation not only reduced the process capacity but also the viral LRV (Table 8.14 and Fig. 8.6) (16). It seems that membrane fouling occurred and great flux decay was generated by the poor viral preparation (Vendor A in Table 8.14). The 3.52 LRV in the final filtrate pool indicates that some particles

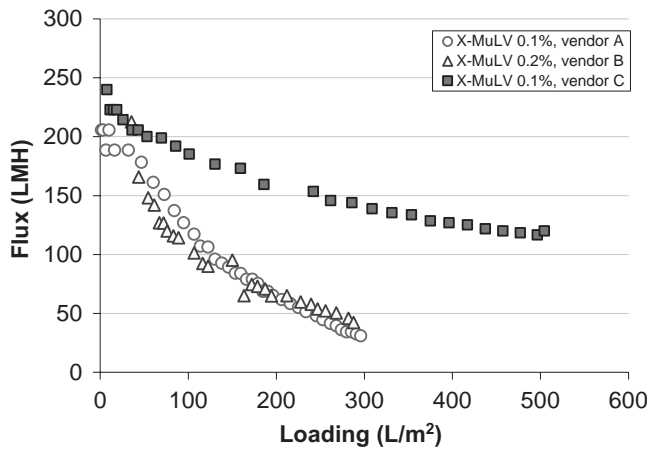


**FIGURE 8.3** Flux decays of five runs with NFP filter without viral spike at the 2000-L scale. Lots 1–5 are represented by squares, diamonds, crosses, triangles, and circles, respectively.



**FIGURE 8.4** Flux decay of NFP filtration with and without spiked virus.





**FIGURE 8.5** Flux decay and process capacity when different virus preparations were used for the determination of filtration capacity.

**TABLE 8.13** Effect of Virus Preparation on LRV Using X-MuLV with NFP Filter

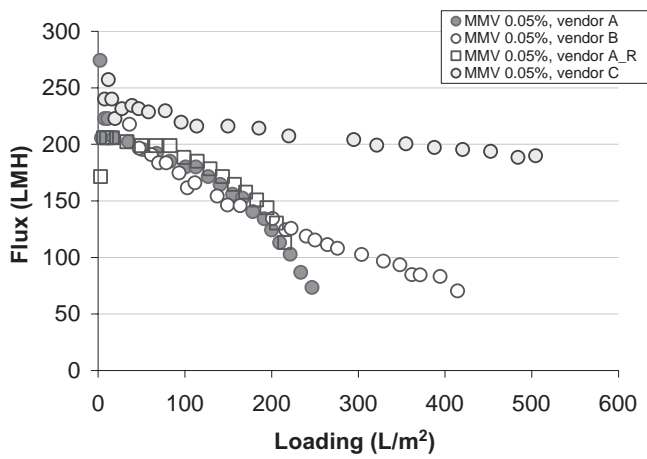
Vendors	Loading, L/m <sup>2</sup>	Flux decay, %	Clearance (LRV)
Vendor A	304	85	≥5.31
Vendor B	304	80	≥3.17
Vendor C	504	50	≥4.87

**TABLE 8.14** Effect of Virus Preparation on LRV Using MMV with NFP Filter

Vendors	Loading, L/m <sup>2</sup>	Flux Decay, %	Clearance (LRV)
Vendor A	246	73.4	3.52
Vendor B	417	65	≥5.47
Vendor C	504	26.1	≥5.95

traveled through the membrane layer (breakthrough) to the pool fraction from the filter’s upper compartment. However, the breakthrough did not occur when the same filter was tested using the virus preparations from Vendors B and C. The preparation from Vendor C appears to have the best filterability with highest process capacity and LRV.

The flux decay curves using the virus preparation by Vendor A (obtained twice) were remarkably different from those of Vendors B and C. In large-volume assays, the LRV values obtained using viral preparations from Vendors B or C were significantly higher than those obtained using the preparation from Vendor A, suggesting that the virus stocks were poorly prepared, either having more viral AGGs or containing more components in the viral media leading to breakthrough.



**FIGURE 8.6** Flux decay comparison of NFP filtration using virus preparations from different vendors.

**TABLE 8.15** MMV Clearance with 20-nm Filters Using Poorly Prepared Viral Stock

Filter	Loading, L/m <sup>2</sup>	Flux Decay, %	Virus Spike, %	LRV
NFP	246.57	93	0.05	3.52
Virosart	374.00	95	0.05	7.27
DV20	82.81	23	0.5	>7.77
Planova	100.00	22	0.5	6.98

Because we observed the breakthrough of MMV particles when using the 20-nm NFP filter and a poorly prepared viral stock, it seemed important to compare the virus clearance performance among all commercially available 20-nm filters (16). Table 8.15 summarizes MMV clearance using four 20-nm filters from different manufacturers. According to the filter vendors’ instructions, a constant pressure of 30 psi was used for NFP and Virosart (Sartorius, Inc., Gottingen, Germany) filters, and 14.5 psi or relatively low pressure was used for Planova (Asahi Kasei Group, Westbury, NY, USA) and DV20 (Pall Life Sciences, Ann Arbor, MI, USA) filters. While no significant flux decay was observed for the latter filters, they were designed for a low load capacity and the filtration time was longer, resulting in low productivity. Taking that into account, the DV20 or Planova filters make less economic sense, although both still achieved excellent virus clearance (15).

Our case study (Table 8.15) suggested that the performance of the NFP filter was poor compared to the similar Virosart filter (16), and breakthrough was even found in the flow-through pool due to membrane fouling. However, when a good virus preparation was used, the highest viral filterability was achieved with X-MuLV ( $\geq 4.87$  LRV at 504 L/m<sup>2</sup>) (Table 8.12) and MMV ( $\geq 5.95$  LRV at 504 L/m<sup>2</sup>) (Table 8.14), and no breakthrough was observed. This

indicates that the NFP filter is highly sensitive and can be used at a high process capacity without membrane fouling when the feed material and virus preparation are satisfactory. Poor process capacity and LRVs will be observed when poorly prepared feed material and viral stocks are used.

As stated above, sizing-based 20-nm filtration is robust and is used in almost all processes involving mammalian cells. Since there should be no virus particles in either the cell culture pool or the intermediate feed pool, the potential virus clearance power of this step must be demonstrated with spiked feed. Virus removal is calculated by subtracting the  $\log_{10}$  of the remaining viruses from the  $\log_{10}$  of the amount of spiked virus, and is dependent on several factors including filter quality, virus preparation, and feed conditions. Filter quality covers the materials used to make the filter (generally polyethersulfone [PES] or polyvinylidene fluoride [PVDF] membranes), the physical structure, which determines the strength to maintain pore size during the filtration, and pore distribution.

Based on our data from several sets of viral filtration experiments, the critical points for the improvement of 20-nm filters in large-scale downstream processes have been defined. Virus stock should be well prepared according to standard methods, which should be indicated by regulatory agencies, and feed materials should be handled with low viscosity and molecular self-association. Once the correct filter material is defined, the current issue of oversized 20nm in the mAb industry will be practically overcome, and high process capacity together with excellent LRVs will be readily achieved.

## 8.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The clearance of viruses from biotherapeutics produced in mammalian cells is critical because of the potential hazard such viruses pose to patients. In case studies with four model viruses, a combination of traditional column chromatography and low-pH inactivation together with disposable systems provides excellent virus removal power. However, virus clearance using disposable systems has attracted more attention because validation is not required, leading to greater cost efficiency, which will be highly sought after in the future of mAb production.

Sizing-based 20-nm filtration is perhaps the most robust clearance system, since it can remove all possible enveloped and nonenveloped mammalian virus particles including all genome types and virion sizes. Virus preparation and feed conditioning remain great challenges to process capacity, resulting in the oversizing of high-cost membrane for the entire industry. A strategic methodology to resolve this issue is needed. Many case studies have demonstrated the popularity of Q membranes in mAb production (32). Q membranes remove viruses as well as AGG, HCP, and rDNA, and mixed-mode chemistry should be considered as a future development to meet the requirements for high-throughput production using two-step purification when processing high-titer

cell cultures. The use of depth filtration for virus clearance is still hotly debated, although depth filtration can consistently remove HCP and rDNA. Indeed, confidence will increase once meaningful and user-friendly integrity or functionality tests are available and can be validated.

In summary, these chromatographic steps and disposable systems now play important roles in the high-throughput downstream processing of mAbs from high-titer cell cultures. The disposable systems not only achieve excellent clearance of potential contaminants such as viruses, HCP, and DNA, but they also offer low costs, manufacturing efficiency, and user-friendliness without the need for cleaning or lifetime and storage validations.

## 8.6 ACKNOWLEDGMENTS

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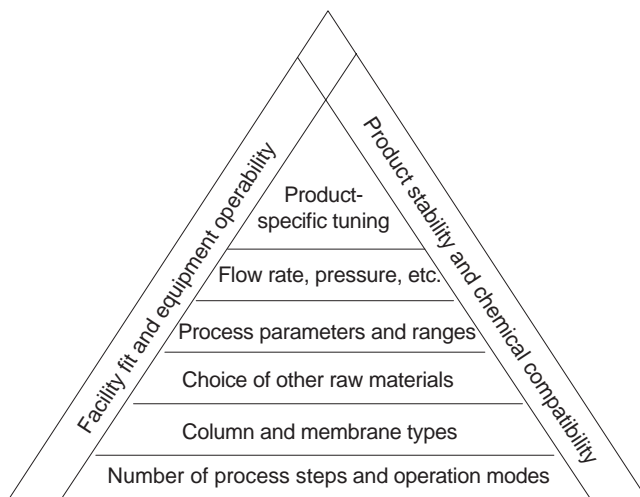
## **DEVELOPMENT OF A PLATFORM PROCESS FOR THE PURIFICATION OF THERAPEUTIC MONOCLONAL ANTIBODIES**

YULING LI, DAVID W. KAHN, OLGA GALPERINA, ERICH BLATTER,  
ROBERT LUO, YALING WU, AND GUIHANG ZHANG

### **9.1 INTRODUCTION**

Many new drug targets have been discovered in recent years and monoclonal antibodies (mAbs) have been developed as therapeutic modalities to target them. This has created a unique opportunity to sharpen the focus of process development strategies, since the structural and biochemical similarity of mAbs allows companies with multiple mAbs in their pipelines to combine their experience from different products and to incorporate the knowledge and improvement into the development of platform processes. The central concept of platform process development for mAbs is the identification and incorporation of generic elements that are applicable to the majority of mAb products, leading to a common framework of purification steps, common chromatography resins, and specific process parameters that could be applied generically to unit operations across various processes (Fig. 9.1).

Benefits may be realized through the use of a platform process both at the early and late stages of a product's development. The early use of a platform process should accelerate process development, facilitating rapid progress toward clinical studies. Further process improvements linked to a deeper knowledge of specific products and their behavior in specific processes can be



**FIGURE 9.1** A hierarchy of purification platform targets.

integrated at later phases of development. Alternatively, companies might choose to adopt an optimized platform to unify divergent purification processes into a common platform at the mid-development stage. In this case, considerations should be based on the stage of development to balance the short- and long-term benefits against regulatory considerations, since certain changes might require an impact assessment, such as a comparability study. The key advantages of the platform approach are shortening of the process development cycle, reduction in raw material inventory through the use of common components, streamlined documentation through the use of templates, facilitation of technical transfer to manufacturing, and the simplification of waste disposal. The platform development approach also allows the focused use of resources in key areas, providing in-depth process knowledge.

The objective of a platform purification process is to integrate optimized chromatography and membrane filtration technologies for efficient production of the target molecule. One can apply orthogonal principles for separation [e.g., affinity, ion-exchange, and hydrophobic interaction chromatography (HIC)] within the process to achieve optimal purification capacity. Ultimately, the platform process should be scalable and reproducible in the manufacturing setting, with acceptable product yield and purity at an acceptable cost.

A typical purification platform process consists of multiple chromatography and filtration steps that aim to take advantage of the biochemical properties of mAbs. Most therapeutic mAbs have a fragment-crystallizable (Fc) region that binds with high affinity to staphylococcal Protein A. Immobilized Protein A-based affinity chromatography is therefore a common component of many platform purification processes (see Chapter 4). Most mAbs have basic isoelectric points that facilitate the use of cation-exchange (CEX) media in



bind-and-elute mode, and anion-exchange (AEX) media in flow-through mode (see Chapter 5), as well as enough surface hydrophobicity to facilitate separation by HIC. However, each mAb has unique biochemical characteristics that make a completely generic platform difficult to envisage (1). Special considerations that may come into play include the molecule's chemical stability, its binding capacity to the platform resin, product degradation pathways that occur during processing, and the distribution of product variants that need to be controlled or removed. With these considerations, as well as specific issues that may arise during implementation at a specific manufacturing facility, the platform process may need minor modifications to achieve complete optimization. The application of these principles to other processes, as well as consideration of new and emerging technologies, might lead to further evolution in the platform process.

This chapter outlines some of the general considerations that must be taken into account when developing a platform mAb purification process, focusing on the suitability of commonly used unit operations and challenges that affect their use. Since most mAb therapeutics are produced in mammalian cell culture, it is important to evaluate process steps for virus clearance by removal and inactivation (see Chapter 8). A platform process provides comparable unit operation steps to build a virus clearance database for future matrix viral clearance validation.

## **9.2 CHROMATOGRAPHY STEPS IN THE PLATFORM PROCESS**

Every purification process can be broken down into a series of unit operations. Operations involving column chromatography may be further classified as either capture or intermediate/polishing steps. The type of chromatography and the operational conditions thus form the basis of a platform process. The following sections outline the alternative capture and intermediate/polishing steps and evaluate their advantages and disadvantages for use in a platform process.

### **9.2.1 Capture Step: General Considerations**

It should be possible to adapt the capture step for multiple products with few changes (or even no changes) in the operational conditions. The role it plays is to remove the antibody from the clarified cell culture feedstream quickly and efficiently while reducing the volume of the feedstream to a level that is manageable in the manufacturing plant. It is highly desirable to reduce host cell protein (HCP), including endogenous proteases, host cell DNA, and endogenous virus or virus-like particles. The capture method also sets the stage for subsequent chromatography steps.

Typical capture steps in a mAb purification process include affinity chromatography (e.g., using either Protein A or Protein G as the affinity ligand),

CEX, or hydrophobic charge induction chromatography (HCIC). The advantages and drawbacks of each are discussed below.

**9.2.1.1 Protein A Affinity Chromatography.** Protein A chromatography is advantageous because it is one of the easiest steps to incorporate into a platform process. The resins bind human IgG1, IgG2, and IgG4 mAbs with extremely high affinity and specificity. The high specificity ensures separation of the target antibody from antibody fragments lacking a functional Fc region, as well as HCP, host cell DNA, and endogenous retroviruses. Other advantages include the resin's tolerance of a wide range of load buffer conditions in terms of pH and conductivity, easily matching typical conditions found in harvested cell culture fluid. This negates the need to condition the column load prior to loading. Protein A columns typically operate at high flow rates and produce high-purity eluate pools with a large reduction in process volume while achieving high product yield. The use of a simple low-pH elution strategy leads naturally into a subsequent virus inactivation step. Finally, the elution step can be performed at low or high conductivity to match subsequent chromatography steps.

Disadvantages of Protein A include the cost of the resins, which are the most expensive media in downstream processing. However, the resins have long lifetimes and can be reused 200 or more times, which helps to reduce the cost per cycle. Many Protein A resins cannot tolerate the high concentrations of NaOH that are used to sanitize other chromatography media, although some can tolerate brief exposure to 0.1 M NaOH, a treatment that generally does not achieve adequate sanitization. IgG3-type molecules do not bind to Protein A (2). In general, the binding capacity of affinity resins is moderate, and one must also monitor leaching of the affinity ligand from the column into the product stream. Finally, the high concentration of mAb following step elution from a Protein A column can result in protein aggregate formation.

**9.2.1.2 CEX Chromatography.** As stated previously, most mAbs have basic isoelectric points and can be captured by CEX over a neutral to a slightly acidic pH range. These resins typically bind mAbs with high capacity, exhibit very low levels of ligand leaching, and are relatively inexpensive. Product elution is achieved using moderate conditions, typically through minor changes in pH and/or conductivity. High flow rates are achievable with only moderate impact on binding capacity with some of the newer resin types. Sanitization can be achieved with high concentrations of NaOH. CEX steps can achieve moderate removal of HCP and DNA with high recovery of the product.

However, load conditioning (pH adjustment and possible diafiltration/dilution) may be required. Typical Chinese Hamster Ovary (CHO) and NS0 (recombinant myeloma cell line) harvested cell culture fluid has a pH between 6.5 and 7.6 with a conductivity of approximately 10–20 mS/cm. Higher conductivity significantly reduces binding capacity, whereas lower conductivity may

result in the retention and copurification of HCP including proteases. CEX columns may potentially produce larger pool volumes than affinity columns.

**9.2.1.3 HCIC.** The advantages of HCIC include the intermediate cost of the resin (somewhere between affinity and ion exchange) and its moderate capacity. Selectivity is also good, but HCIC resins are not as selective as affinity media. HCIC can also be used as a virus clearance step, and performs just as well as other robust steps. The binding of a mAb to an HCIC resin occurs in the pH range 6.8–7.5, with extremely low levels of leaching. Cleaning is possible with high concentrations of NaOH. Nonspecific binding can be reduced with high conductivity and/or with lower pH washes. Elution of the mAb is achieved by reducing the pH below 4.5. The elution pH can affect both the elution volume and the product purity. The major contaminant observed in HCIC is the light chain dimer (unpublished results).

It is difficult to assess the disadvantages of HCIC because experience with the resin is limited and the resin shows a lower selectivity compared with conventional affinity resins.

**9.2.1.4 Overview of Capture Resin Platforms.** Of the resins described above, Protein A is typically the easiest to implement in a mAb platform process with minimal process development. Loading conditions can be standardized across a panel of different antibodies. The high specificity of the resin for antibodies ensures a very robust first step. These benefits need to be balanced against the high resin cost, the inability to use NaOH-based cleaning regimens, and the addition of residual Protein A to the product stream. HCIC could be used as an alternative based on the ease of implementation. Typically, this step would not require load conditioning, but additional development to maximize binding may be required. Wash and elution conditions would need to be optimized individually. Sanitization regimes can be similar to other base-stable resins. The use of a CEX capture step would require more individual development work but could offer higher binding capacity while maintaining lower costs and more rigorous sanitization schemes. If the panels of antibodies to be used in the platform process share similar isoelectric points, it should be feasible to use a platform process beginning with CEX with moderate development. For example, a fixed load adjustment for capture, a mild wash that can accommodate a range of binding affinities, and sharp pH elution could be applied to a group of antibodies with very similar biochemical properties. Tuning of conditions for each antibody would generate improvements in purification, yield, and robustness.

## 9.2.2 Intermediate/Polishing Steps

The type and number of these steps depend on the capture step, the level of impurities in the load material (product- and process-related impurities), and the amount of virus clearance required. It is important to use orthogonal

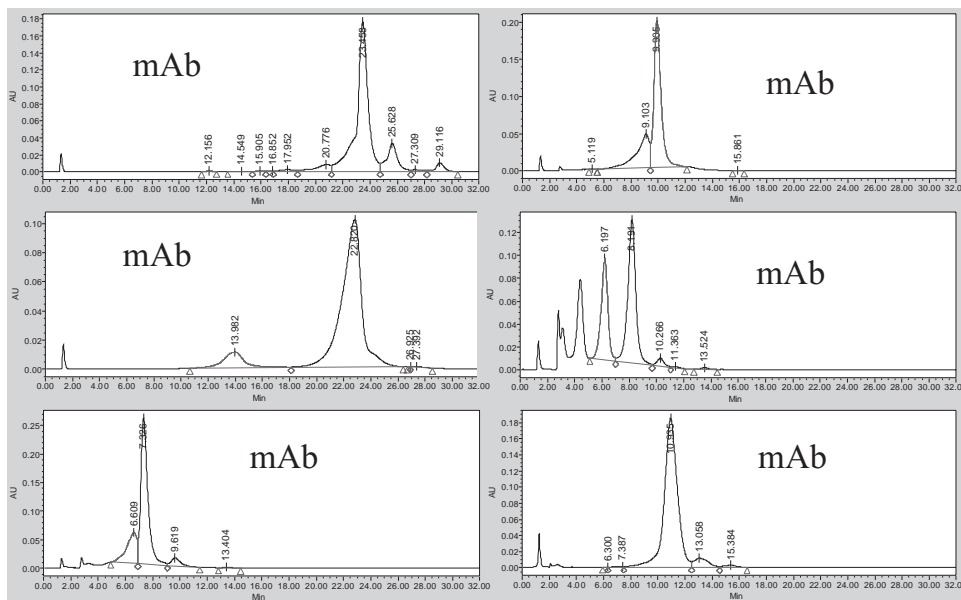
purification steps wherein fractionation is achieved using distinct modes. If an affinity column is used for capture, then the use of a subsequent ion-exchange or HIC/HCIC column would be beneficial. If CEX is used for capture, it is usually followed by AEX or HIC. Affinity chromatography is not commonly used as an intermediate or polishing step in mAb purification. Employing a different chromatographic principle for each step helps to provide a robust purification process.

**9.2.2.1 CEX Chromatography.** If CEX follows a Protein A capture step, elution from the affinity column should be carried out in a low-conductivity buffer. The low-pH elution buffer can be adjusted to match the binding conditions required for the CEX column. If, on the other hand, the capture step involved HIC or HCIC, then the conductivity of the elution pool would need to be adjusted by dilution before the CEX step.

Depending on the purification challenge, a CEX step in the second position can be tuned to carry out specific separations. For example, the clearance of process-related impurities such as residual Protein A, HCP, and DNA can be achieved by step elution, whereas for finer separations, such as product-related impurities, gradient elution can be used instead. Even at process scales, excellent separation is possible. The more that is asked from the step, the more individual tuning will be required for specific products. A comparison of six different antibodies applied to the same CEX high performance liquid chromatography (HPLC) column running the same gradient method is shown in Fig. 9.2. While this degree of separation is not possible in process scale columns, a gross separation of regions of the chromatogram is feasible.

**9.2.2.2 AEX Chromatography.** AEX after capture is performed in either flow-through or bind-and-elute mode, depending on the isoelectric point of the antibody. If the step is used for the clearance of viruses and/or residual contaminants, flow-through mode is extremely efficient and easy to apply as a platform step. Thus, the pH and conductivity is set so that the antibody panel can flow through the column while viruses, HCP, endotoxins, and DNA bind to the resin. Similar separations can be performed in bind-and-elute mode with the added complexity of wash and elution steps. Again, it is necessary to align the requirements of the polishing step with the pH and conductivity of the capture pool.

**9.2.2.3 HIC.** HIC after capture is advantageous because it is easy to achieve the correct conductivity and pH by load conditioning. Elution is accomplished simply by stepping to a lower conductivity. HIC can be implemented for the removal of high-molecular-weight (HMW) mAb aggregates, viruses, HCP, and leached Protein A (3). An ultrafiltration/diafiltration (UF/DF) step or high dilution is likely to be necessary following HIC elution. This is convenient if HIC is the last chromatography step before exchange into formulation buffer.



**FIGURE 9.2** Chromatograms from six different mAbs processed on the same CEX column using an identical method.

**9.2.2.4 Assessment of Polishing Resins.** The selection of the capture resin has a strong influence on the best choice for an intermediate purification/polishing strategy. Of the various media (excluding affinity resins), HIC resin may be the easiest to implement in a platform process because of the ease of load conditioning. However, if a third chromatography step is required, an ion-exchange step can readily be incorporated before HIC.

### 9.3 VIRUS INACTIVATION

To ensure product safety, a dedicated virus inactivation step is required in every mAb purification process (see Chapter 8). Virus inactivation can be accomplished by treatment with solvents and detergents, or by exposure to low pH conditions for a short period of time. As discussed earlier, affinity columns typically elute at low pH and mAbs tend to be stable for a short time in low-pH buffers; this is often chosen as a virus inactivation step. A drawback to this approach is the formation of low levels of precipitate that are often non-product related. Even non-product-related precipitates are generally undesirable since additional filtration is required, thus extending the process duration. The precipitates generally comprise HCP, DNA, and/or denatured mAb, and if maintained in a soluble state, they are easily removed in subsequent purification steps. Buffer conditions in the step preceding low-pH

inactivation should therefore be optimized with this in mind. In some cases, optimization with additives such as salts can prevent precipitation.

## **9.4 UF/DF PLATFORM DEVELOPMENT**

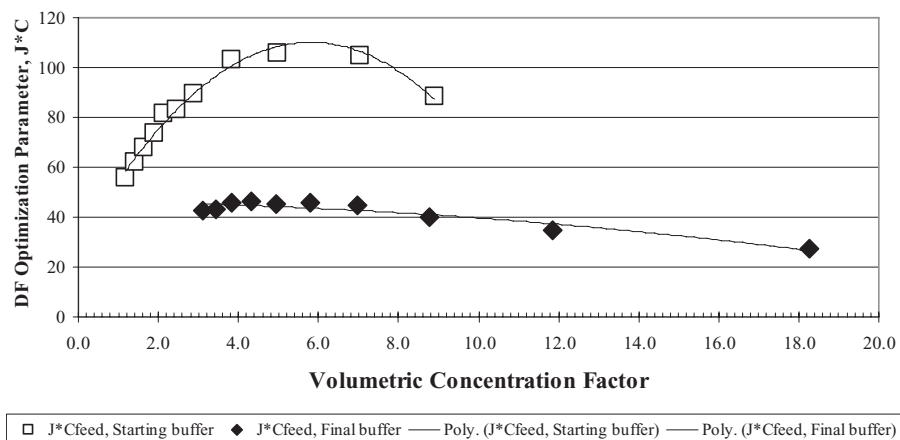
The UF/DF step is used for product concentration, buffer exchange, and formulation. It can be implemented at any stage in the downstream process when concentration, buffer exchange, or formulation is required. For products with a low final protein concentration, a simple UF/DF process is adequate. However, for products with high final concentrations, gelation on the membrane surface can occur when the concentration is high, which can reduce permeate flux dramatically. This in turn will prolong the process time. Therefore, in situations with high product concentrations, a UF1/DF/UF2 approach can be used to optimize process performance. The UF/DF step could well be one of the most complicated steps in a mAb purification process.

### **9.4.1 Use in Platform Processes**

A UF/DF membrane that works for one antibody process is often a good choice for other mAbs. This saves the time required to screen new membranes and to integrate them into the process. Operational parameters such as trans-membrane pressure (TMP) and cross-flow rate from one process are often good starting points for the development of a new process even though further optimization may be required. Process data such as DF volume and membrane cleaning strategy should be highly adaptable to a new process with minimal changes (or no changes) in the conditions. In addition, the methodology employed to determine the optimal protein concentration used during DF can be applied to a platform process strategy.

### **9.4.2 Challenges**

It is often necessary to administer large amounts of a mAb to achieve a sufficient therapeutic effect, but delivery is occasionally limited by injection volume, particularly for drugs administered by subcutaneous injection. Therefore, it is necessary to produce antibody products formulated at high concentrations to deliver an adequate product in small injection volumes. Reliable, scalable processes must be developed to produce such high concentrations without compromising product quality. However, in large-scale commercial operations, this may be a challenge. Proteins become highly concentrated during the UF2 phase, making foaming an issue at high flow rates. Foaming in the retentate tank might cause protein aggregation and might interfere with the accuracy of volume measurements, potentially resulting in the overconcentration or underconcentration of the product. High protein concentration in the UF/DF operation also presents a challenge when cleaning the UF/DF membranes, possibly contributing to a higher bioburden in subsequent pools.



**FIGURE 9.3** Optimization parameter vs. volumetric concentration factor.

### 9.4.3 Application Examples

In UF/DF process development, it is critical to identify the optimized point to end UF1 and to start DF. The following methodology can be applied to a platform process strategy (4). A parameter called “optimization factor, JC” was used to decide the UF1 end concentration. Here  $J$  is the permeate flux, and  $C$  is the retentate concentration. A maximum value of JC as a function of “volumetric concentration factor (VCF)” is sought to optimize the process. Here VCF is defined as the ratio of the initial volume to the final volume.

Figure 9.3 shows one approach that can be used to obtain the JC corresponding to the optimal end time for UF1. In the figure, JC was plotted against VCF for both the DF starting buffer and the DF ending buffer. The VCF corresponding to the maximum JC is  $VCF_{DF}$  (the VCF for starting DF).

## 9.5 PLATFORM DEVELOPMENT: VIRUS FILTRATION AND BULK FILL

Most antibody purification processes implement a dedicated filtration step for the removal of viruses to ensure a robust clearance capability. This step is readily adapted into platform processes.

### 9.5.1 Virus Filtration in Platform Processes

The application of platform technologies to the virus filtration step can be time saving and extremely beneficial. The development challenge for this step is that virus filters, especially the small-pore nanofilters, are very sensitive to particles and impurities in the feedstream. These impurities reduce filter capacity dramatically. Therefore, the main objective is typically to keep the step at

the same position in the platform process to maintain a similar feedstream with regard to buffer composition, protein concentration, and impurity profiles. With a similar feedstream, the capacity of the virus filter and the processing time for the step is predictable based on previously developed knowledge. Previous knowledge such as load storage conditions, filter type/source, filter capacity, operation flow rate and/or pressure, and validation strategies can be useful for the development of new processes. Process development time can be minimized for an early-phase product as long as the filter capacity is similar to that of previous processes. For a late-phase product, process characterization could be needed for a better understanding of the impact of process parameters and their interactions. In addition to process development, a platform virus removal validation strategy and virus validation data from similar antibody processes can be applied to new processes. Overall, platform technology is applicable to the virus filtration step and should be considered as part of a mAb process development strategy.

### **9.5.2 Bulk Fill in Platform Processes**

The bulk fill step is critical and yet straightforward as compared to other purification steps. Because of its relative simplicity, the step is readily incorporated into a platform process. The purpose of this step is to filter the product aseptically in preparation for fill and finish.

Normally, the same type of filters can be used for a new process if the filter selected for the platform provides optimal capacity. In general, the filter capacity is product specific and needs to be investigated. Previous development knowledge concerning filter capacity and operation flow rate/pressure in relationship to protein concentration and formulation buffer composition certainly helps in the development of a new platform process. If the filter size and setup used for the process are different, additional development may be required. However, if similar, platform technologies will be valuable and applicable.

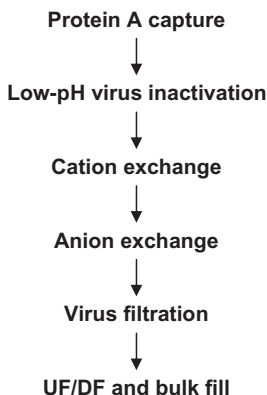
## **9.6 EXAMPLES OF PLATFORM PROCESSES**

In this section, we outline several examples of potential platform processes (5). The combination of chromatography methods makes for many permutations, but the ultimate choice will depend on the purification challenge (i.e., the cell line, the levels of process- and product-related variants, and virus clearance requirements).

### **9.6.1 Example 1: Three-Column Protein A-Based Process**

The first example is a common Protein A-based antibody purification process (5). It covers a broad range of antibodies and conditions, and can be used as a reference point to evaluate other platforms (Fig. 9.4).





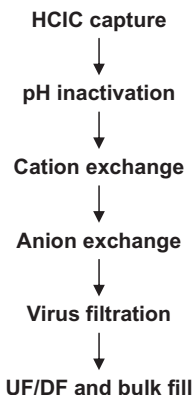
**FIGURE 9.4** Common antibody purification process, example 1: a three-column Protein A-based process.

The recovered cell culture supernatant (pH 6.0–8.0) is passed through a Protein A column equilibrated using a similar buffer. The column is washed with one or more buffers to remove nonspecifically bound HCP, DNA, and other impurities. The column is eluted by reducing the pH to 3.0–4.0, usually with a glycine, acetate, or citrate buffer. The elution step will bring the pool concentration close to the pH range where low-pH virus inactivation can be achieved (approximately pH 3.0–4.0). After virus inactivation, the pool is adjusted for CEX chromatography. Load conditioning will depend on the elution conditions for the capture column, the isoelectric point of the antibody, and the desired binding capacity on the column. After loading and washing to remove nonbound materials, the target antibody can be eluted by increasing conductivity, pH, or both, in the form of a step or gradient. The pH and conductivity of the pool may have to be adjusted for the subsequent AEX step, which is carried out in either the flow-through or the bind-and-elute mode depending on the isoelectric point of the antibody. Virus filtration could be performed before, or after, the AEX step. The antibody is then concentrated and exchanged into formulation buffer by UF/DF prior to bulk filtration.

### 9.6.2 Example 2: Three-Column HCIC-Based Process

By eliminating Protein A from the process, the high cost of the affinity resin is avoided, and it is no longer necessary to remove leached Protein A. Furthermore, caustic cleaning solutions can now be used. In the second example, Protein A is replaced with HCIC. Alternatively, Protein A could be replaced with a small-molecule dye with IgG specificity (6) (Fig. 9.5).

In this example, recovered cell culture supernatant (pH 6.0–8.0) is passed over an HCIC column equilibrated in a buffer of similar pH and optionally containing a high concentration of NaCl. The column is washed with one or more buffers to remove nonspecifically bound HCP, DNA, and other



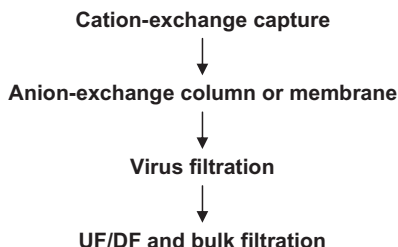
**FIGURE 9.5** Common antibody purification process, example 2: a three-column HCIC-based process.

impurities. The column is eluted by decreasing the pH to approximately 4.0–4.5 usually with a glycine, acetate, or citrate buffer. The elution step brings the pool concentration close to the pH range where low-pH virus inactivation can be carried out (approximately pH 3.0–4.0). After virus inactivation, the pool can be adjusted for CEX chromatography. Load conditioning will depend on the elution conditions for the capture column, the isoelectric point of the antibody, and the desired binding capacity on the column. After load and wash steps, the antibody can be eluted by increasing the conductivity, pH, or both, in the form of a step or gradient. The pH and conductivity of the pool may need to be adjusted for the AEX step. This step can be performed in flow-through or bind-and-elute mode depending on the isoelectric point of the antibody. Virus filtration can be performed before or after the AEX step. The antibody is then concentrated and exchanged into formulation buffer by UF/DF prior to bulk filtration.

### 9.6.3 Example 3: Two- or One-Column Process without Protein A

By leveraging the power of CEX as the capture step, it is possible to reduce the number of columns *and* eliminate Protein A. If an AEX membrane is used to replace a column, it may be feasible to achieve mAb purification through a one-column process (7) (Fig. 9.6). The absence of a Protein A column eliminates the need to remove leached Protein A further downstream. It should be noted that to maintain robust clearance of viruses and to achieve high product purity with few chromatography steps, the starting material must have a low initial virus particle count and must contain very low amounts of product variants.

In this example, the cell culture supernatant should have a conductivity low enough to load directly onto the CEX column. A dilution step could be performed if necessary. The column is then washed and eluted either by step or



**FIGURE 9.6** Common antibody purification process, example 3: a two- or one-column process without Protein A.

by gradient elution. Low-pH virus inactivation could be performed in a variety of positions in this scheme. One interesting possibility is to perform the low-pH inactivation in the presence of cells, before harvest. The resulting precipitate can readily be removed during the harvest step. After CEX, the pH and conductivity may need to be adjusted before loading onto the AEX column or membrane. The primary purpose of this step is virus clearance. The pool can then be passed through a virus filter and concentrated in formulation buffer by UF/DF prior to bulk filtration.

## 9.7 DEVELOPING A VIRAL CLEARANCE DATABASE USING A PLATFORM PROCESS

The major components of a robust virus safety strategy for antibody production in mammalian cell lines include the control of raw materials, the characterization of cell lines, good manufacturing practices, and evaluation of the downstream process units designed to clear or to inactivate viruses.

The objective of the virus safety risk assessment performed at the end of each stage of process development is to evaluate the production cell line as well as the virus clearance capacity of the production process in relation to the dose and the clinical study design. The majority of antibodies in current production use well-characterized cell lines such as CHO or NS0. The main challenges that industry faces in the area of virus safety are the relatively high endogenous retroviral loads, which are especially notable in the NS0 cell lines, combined with relatively high doses that are usually required for effective treatment using mAbs. Typical retroviral particle counts in unprocessed bulk, estimated using a transmission electron microscopy (TEM) assay, are in the range of  $10^8$ – $10^{11}$  particles/mL. In consideration of other factors, such as process performance and dose requirements, a typical mAb purification process would need to remove approximately 18–20 logs of retrovirus.

Virus clearance studies are expensive and time-consuming. Any reduction in the scope or number of studies resulting from use of data derived from platform processes would be desirable. The use of upstream and downstream

**TABLE 9.1 Summary of Virus Clearance Results for the Downstream Antibody Processes**

Product	Capture	Low-pH Inactivation	AEX	HIC	Virus Filter
mAb 1	4.18	≥4.44	≥5.12	≥5.25	≥3.57
mAb 2	4.49	≥5.94	≥4.66	3.74	≥3.93
mAb 3	4.87	≥4.65	≥6.03	≥5.27	≥4.45
mAb 4	3.60	≥4.01	4.01	≥4.4	≥4.22
mAb 5	3.84	≥3.79	≥5.10	≥5.1	≥6.18
Average	4.35 ± 0.44	≥4.57 ± 0.84	≥4.98 ± 0.84	≥4.75 ± 0.67	≥4.95 ± 1.07

platform processes may facilitate the development of an in-house virus clearance database that would demonstrate the effectiveness of each unit operation in terms of virus clearance over a defined range of values for important process parameters (8, 9).

A typical virus clearance study for a mAb product entering clinical development focuses on retroviral clearance using a model virus such as xenotropic murine leukemia virus (X-MuLV). The evaluation of X-MuLV clearance typically covers several orthogonal unit operations. Table 9.1 lists virus clearance results achieved in various unit operations, expressed as log reduction values (LRVs).

Each of the steps provides reliable retrovirus clearance with an LRV of at least 3.5 and usually >4. Low-pH viral inactivation in particular appears to be a valuable step readily incorporated into platform processes. The buffer composition, as well as the ionic strength of the solution, does not appear to have much influence on the effectiveness of the low-pH inactivation step.

Among other advantages, a platform process allows the creation of an in-house virus clearance database. The database should capture the efficiency of virus clearance over a range of conditions and parameters. Evidence of effective virus clearance over a range of conditions helps to demonstrate the robustness of particular unit operations.

## 9.8 SUMMARY

The use of a platform process for mAb purification offers numerous potential advantages over traditional “one-step-at-a-time” approaches. In addition to accelerating process development while minimizing the resources required, cost savings can be realized through use of common raw material components. There may also be benefits in terms of “process fit” when implementing multiple processes into a single facility, if the processes have been designed from a common platform. Several steps commonly found in mAb processes seem particularly amenable for use in a platform process, including Protein A chromatography, low-pH virus inactivation, flow-through AEX chromatography,

virus filtration, UF/DF, and bulk filtration. Finally, in regard to virus clearance requirements, the use of a platform approach enables the creation of sizable databases of valuable information that help characterize clearance steps. These data may allow generic claims of virus clearance, assuming one stays within the process parameters contained in the database. In conclusion, with the continued widespread success of mAbs as biopharmaceuticals, the continued use of the platform approach in process development will offer continued benefits to users in the future.

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# 10

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## **ADVANCES IN TECHNOLOGY AND PROCESS DEVELOPMENT FOR INDUSTRIAL-SCALE MONOCLONAL ANTIBODY PURIFICATION**

NUNO FONTES AND ROBERT VAN REIS

### **10.1 INTRODUCTION**

Advances in purification technology and process development are required to meet the increasing challenges in the industrial-scale recovery of monoclonal antibodies (mAbs). Increasing cell culture titers and the pressure for economical manufacture create a need for higher binding capacities and an opportunity for new cost-effective technologies. This chapter reviews advances in technology and process development for the industrial-scale purification of mAbs, focusing on cation-exchange (CEX) chromatography, a technique used in most industrial mAb purification processes, and on high-performance tangential flow filtration (HPTFF), an emerging purification technology. We provide a review of industrially significant developments made by the authors and colleagues at Genentech in collaboration with leading suppliers of purification raw materials. We show how an improved understanding of the separation mechanisms leads to improved technologies, efficient process development methodologies, and cost-effective manufacturing processes.

Section 10.2 provides a brief overview of Genentech's current affinity mAb purification platform. We discuss how the limitations imposed by the performance and cost of current raw materials create the need for advances in purification technology and process development.

Section 10.3 focuses on the contributions made by Genentech and GE Healthcare to the fundamental understanding of ion-exchange phenomena, and its implications for industrial process and new technology development. It has been demonstrated that the dynamic binding capacity (DBC) in CEX chromatography can, in contrast to the traditionally described behavior, increase with increasing salt concentration and display a maximum at intermediate ionic strengths, corresponding to a balance between equilibrium and mass transfer effects mediated by a protein exclusion mechanism (1, 2). Although hindered diffusion has been known to limit mass transfer in bead-based separation techniques (3–5), Genentech and GE Healthcare have revealed how protein exclusion phenomena can lead to the atypical dependence of the DBC on ionic strength with mAbs in CEX media. This section discusses how knowledge of the exclusion mechanism can be leveraged to improve process development methodologies and to design high-capacity ion-exchange media.

Section 10.4 reviews recent developments in HPTFF, focusing on contributions made by Genentech in collaboration with Millipore Corporation. We have developed a new membrane that eliminates the requirement for a cocurrent filtrate loop, enabling the use of the entire base of ultrafiltration systems in the industry. Finally, Section 10.5 presents an example of the integration of the technologies discussed in the previous sections, into a nonaffinity two-chromatography step, cost-effective mAb purification process. We present a brief discussion of the challenges and opportunities of this new platform based on a CEX capture step followed by an anion-exchange (AEX) membrane adsorber and HPTFF as a final step.

## **10.2 AFFINITY PURIFICATION PLATFORM**

### **10.2.1 Overview**

The physicochemical similarities between mAbs and the need to develop manufacturing processes for a broad mAb-based clinical pipeline have led the major biopharmaceutical manufacturers to adopt a platform approach to purification (6) (see Chapter 9). This concept was made possible by the high selectivity of Protein A affinity chromatography for mAbs. Protein A is typically the first step due to its ability to capture the product from the unconditioned harvested cell culture fluid (HCCF) with purities greater than 99% (see Chapter 4). Shukla and colleagues (6) have recently presented an in-depth discussion of the pros and cons of platform approaches to the purification of mAbs. The major drivers for the adoption of a generic template for mAb purification come from the savings in time and resources required to conduct process development. In addition, other aspects such as facilitated alignment of cross-functional groups throughout the company (6), lower raw material costs due to better deals with a restricted number of raw material suppliers,

opportunity to apply modular approaches to process validation, among others, contribute to the attractiveness of a platform approach.

### 10.2.2 Standard Purification Sequence

At Genentech, the standard purification sequence begins with the harvest of the cell culture fluid by centrifugation and depth filtration followed by Protein A affinity chromatography. This step removes host cell proteins (HCPs), DNA, viruses, and small molecules such as cellular metabolites and cell culture media components. The product is eluted in acidic conditions and held at low pH for a defined period of time for retroviral inactivation. After conditioning, this pool is loaded onto a bind-and-elute CEX step primarily for the removal of aggregates, product variants, and process-related impurities such as leached Protein A. In addition, this step provides further removal of HCP and DNA. The CEX pool is then loaded onto a retrovirus or parvovirus filter and conditioned prior to loading onto a flow-through AEX step, mainly for viral clearance and final removal of HCP and DNA. The conditioned AEX pool is concentrated and exchanged by ultrafiltration into a suitable buffer, and then formulated with the appropriate excipients.

### 10.2.3 Challenges and Opportunities

Despite the high selectivity and platform-enabling properties of Protein A affinity resins, its price, in dollar per liter of resin or in dollar per gram of purified mAb, is an order of magnitude higher than that of conventional nonaffinity chromatography media, accounting for up to a third of the overall raw material costs. This is a major challenge to the development of cost-efficient manufacturing processes. Other disadvantages of Protein A include the intolerance of most Protein A ligands to the caustic regeneration and sanitization used in conventional chromatography media. This has been partly overcome with MabSelect SuRe from GE Healthcare, which is composed of an alkali-stabilized Protein A-derived ligand. Nevertheless, polypeptide-based ligands will always display some sensitivity to base and a potential drop in resin capacity with regeneration cycles. Another limitation of Protein A affinity chromatography is the low DBC compared to that of ion-exchange chromatography. Despite recent reports of capacities up to 50 g/L (7), Protein A is intrinsically limited to a lower maximum theoretical binding capacity due to the larger volume occupied by the Protein A ligand compared with that of ion-exchange media. Equilibrium capacities of up to 65 g/L have been reported (8, 9). This value is less than half of that achievable with cation exchangers. Moreover, the strong mAb/Protein A interaction is likely to limit intraparticle mass transfer to a greater extent than that observed in ion-exchange media. This may be partly overcome by using larger-pore-size beads. However, this results in an overall lower surface-to-volume ratio and in a consequent drop in the maximum binding capacity. In addition, the requirement for a specific spatial orientation



between the mAb and the Protein A ligand may limit the maximum number of mAb molecules that can be bound per unit surface area. Other disadvantages of Protein A include the leaching of ligand into the process pool and potential product stability issues associated with the low-pH elution.

Despite its virtues and all the efforts to mitigate its limitations, Protein A is still the most expensive chromatographic step in the purification scheme, and is thus the prime target for step elimination and reduction of cost of goods (COG). Follman and Fahrner (10) have demonstrated the feasibility of a non-affinity three-chromatography step mAb purification process. An alternative approach to the use of Protein A consists of using a combination of chromatographic and nonchromatography purification technologies such as precipitation, flocculation, crystallization, extraction, and HPTFF (11) (see Chapters 5, 14, and 15). Another approach to nonaffinity mAb purification consists of a two-chromatography step process, with a CEX capture and another chromatography polishing step followed by high tangential flow filtration (12, 13). Despite the relative success in the use of CEX resins for mAb capture from cell culture fluid with HCP removal comparable to that of Protein A (13), ion exchangers can be further optimized for this specific application.

Despite reported capacities in excess of 150 g/L with mAbs in CEX resins, these are obtained with relatively pure loaded samples such as Protein A pools. When HCCF is loaded onto the resins, the maximum observed capacities are typically lower, in the order of 80 g/L (13), due to competition with HCP. Therefore, improvements in CEX chromatography capacity and selectivity are desired to enable efficient nonaffinity capture of mAbs from HCCF.

## **10.3 ADVANCES IN CEX PURIFICATION OF mAbs**

### **10.3.1 Overview**

CEX is commonly used in the industrial purification of mAbs (2, 6), providing removal of HCP, DNA, leached Protein A, viruses, and product variants.

Commercially available cation exchangers can be divided into low-capacity and high-capacity resins, according to their DBC for mAbs. At industrially relevant load residence times, observed maximum DBCs are in the range of 50 g/L with low-capacity resins such as SP Sepharose Fast Flow (SP FF) and POROS 50HS, and about 100 g/L with high-capacity resins such as SP Sepharose XL (SP XL), Capto S, and Fractogel SE Hicap. Low-capacity resins tend to display superior purification performance and have been extensively implemented for industrial applications. Adoption of high-capacity CEX resins has been slow due to insufficient purification performance, particularly in the removal of HCP at high load capacities. New cation exchangers, with DBCs in excess of 100 g/L and improved purification performance, will be required for the industrial-scale purification of increasing batch sizes of mAbs. This section provides a review of contributions made by Genentech in collabo-

ration with GE Healthcare in the understanding of mAb transport in CEX media, which resulted in advances in CEX process development and in a new generation of high-capacity high-performance CEX resins for mAb purification.

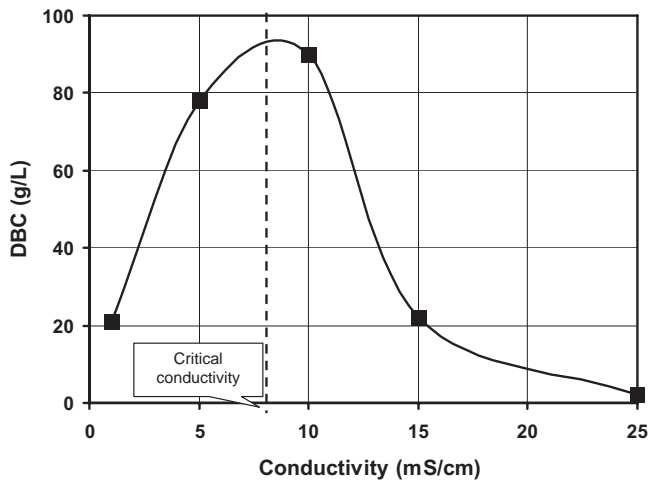
### 10.3.2 High-Capacity CEX

Protein ion-exchange processes are based on the electrostatic interaction between oppositely charged amino acid ionizable groups and resin ligands. Maximal DBC results from an optimal combination of fast protein transport, large binding surface-to-volume ratio, and efficient protein–ligand binding. This can be achieved through manipulation of resin and process design parameters. Traditional resin design parameters include particle and pore size, pore connectivity, ligand density, and the chemistries of the backbone, spacer, and ligand. More recently, CEX binding capacity has been increased by an improvement in the utilization of the pore volume for protein binding by coupling the charged ligand to flexible polymer extenders that fill up the pore space, usually dextran, grafted onto the rigid porous matrix (14). Key process parameters include load pH, ionic strength, residence time, and protein concentration. The strength of ionic interactions is directly proportional to the magnitude of the charges and is inversely proportional to the ionic strength of the medium. As a consequence, the binding capacity in CEX is expected to increase with increasing opposite protein and resin charge density and decreasing ionic strength. Recent work has demonstrated that this common assumption can be wrong with mAbs, particularly on high-capacity CEX resins (2). In fact, it has been shown that there are conditions in which capacity increases with increasing ionic strength and decreasing protein charge. This has been demonstrated with three different mAbs with two different CEX resins. DBC displayed a bell-shaped curve dependence on ionic strength, with the maximum capacity being achieved at an intermediate salt concentration (Fig. 10.1).

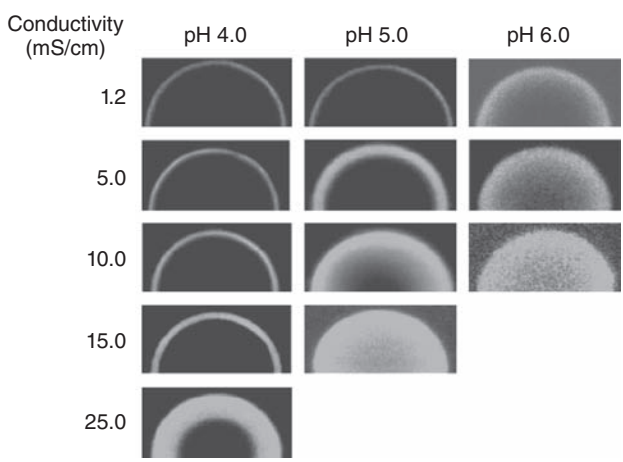
### 10.3.3 An Exclusion Mechanism in Ion-Exchange Chromatography

A protein exclusion mechanism was postulated to explain the atypical dependence of DBC on conductivity with mAbs in CEX resins (1, 2). Proteins initially binding to the outer pore regions sterically and electrostatically hinder the diffusion of other protein molecules that may be excluded from transport into the resin, significantly limiting the DBC. In a study of the exclusion mechanism in conventional CEX, Harinarayan and colleagues (2) provided evidence that exclusion effects are influenced by the strength of protein–protein and protein–resin electrostatic interactions. In the same study, direct visualization of protein transport by confocal microscopy supported the exclusion mechanism (Fig. 10.2).

Two domains were observed in DBC trends as a function of pH and conductivity obtained with a mAb in both SP FF and SP XL. The unexpected



**FIGURE 10.1** Dynamic binding capacity (DBC) as a function of conductivity. The mAb is in SP XL at pH 5.0. The vertical dashed line indicates the critical conductivity, i.e., the conductivity value corresponding to the maximum DBC value at a given pH and load residence time.



**FIGURE 10.2** Confocal microscopy images by Anders Ljunglöf—GE Healthcare, recorded after 5 min of uptake of a mAb to SP XL during finite bath adsorption, pH 4.0, 5.0, 6.0; conductivity at 1.25, 5.0, 10.0, 15.0, and 25.0 mS/cm. From reference 2, Copyright © 2006 by John Wiley & Sons. Reprinted by permission of John Wiley & Sons.

increase in DBC with increasing conductivity observed in the first domain was explained by the exclusion mechanism. At very low salt concentrations, the protein binds strongly to the resin, which is traditionally regarded as a requirement for high capacity. However, in this strong binding condition, protein–protein steric and electrostatic hindrance appears to slow down protein diffusion

and to prevent full utilization of the bead volume within a reasonable residence time. Increasing the ionic strength shields protein–protein repulsion and attenuates the electrostatic-based exclusion between proteins bound at the pore entrance and new incoming protein molecules, enhancing protein transport into the core of the resin bead, resulting in greater bead volume utilization and higher DBC. The second domain displays the expected decrease in equilibrium capacity with increasing ionic strength, which is explained by the decreasing electrostatic interaction between the protein and the resin with higher salt concentration. In this region, despite the unhindered protein transport and efficient utilization of the bead volume, weaker protein–resin interactions limit the extent of protein binding due to low equilibrium capacity. The maximum DBC at a given pH and load residence time is thus obtained at an optimal conductivity, defined by Harinarayan and colleagues (2) as the critical conductivity that, for a particular residence time, provides an optimal balance between exclusion-limited protein transport and equilibrium binding capacity.

Others had also recognized the importance of hindered diffusion due to adsorbed protein and of atypical effects of ionic strength on protein mass transfer in ion exchange (5, 15). Chang and Lenhoff (16) investigated the effect of ionic strength on dynamic uptake rates of lysozyme in preparative cation exchangers and noted a clear trend of increased effective protein diffusivity with increasing salt concentration. They postulated that this was unlikely to be related with direct salt effects on protein diffusion but was due to a salt modulation of protein–protein and protein–surface interactions. The authors pointed to several possible mechanisms including, among others, retarded surface diffusion due to stronger binding at lower concentrations, and an increase in the repulsion between free, diffusing protein molecules and their adsorbed counterparts at lower salt concentrations, a concept similar to protein partitioning and transport in a pore of like charge, resulting in a reduction in diffusivity with increasing repulsion.

In a confocal microscopy study of the effect of ionic strength on lysozyme uptake in SP FF (17), the authors noted that in contrast to the sharp fronts observed at low ionic strength as the protein penetrates into the particle, the intraparticle profiles were unexpectedly diffuse at a higher ionic strength of 100mM and that the uptake reached completion much more quickly than at low ionic strength. The effective pore diffusivity increased almost an order of magnitude as the ionic strength was increased. The authors suggested a possible exploitation of this acceleration in macroscopic uptake rates with increasing ionic strength to improve column loading efficiency in large-scale operations. In a different study (18), the authors have also alluded to a trade-off between equilibrium (static capacity) and protein transport in the lysozyme–SP FF system. They noted that the capacities increase but uptake rates decrease with decreasing ionic strength, realizing that optimization of the uptake rate could be achieved by tuning the ionic strength.

There is a consensus that intraparticle protein transport represents the main resistance controlling protein uptake in preparative separations (19). Two limiting models are commonly used to describe protein uptake: pore diffusion,

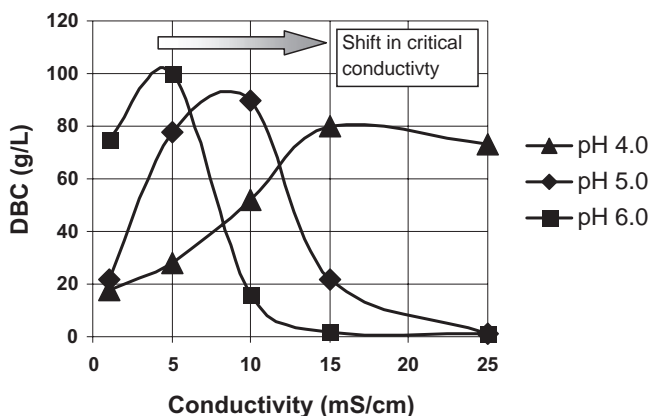
in which only free protein in the pore contributes to transport, and homogeneous or surface diffusion, in which the protein flux is determined by the gradient in the total protein concentration, free or adsorbed. Studies have associated the increase of uptake rates with increasing ionic strength to a transition from pore to homogeneous diffusion (19). Electrokinetic mechanisms may also be involved in the regulation of protein diffusion in ion exchange (17).

Another mechanism, consistent with the atypical dependence of DBC on ionic strength with mAbs in ion-exchange resins, is a variant of the random sequential adsorption (RSA) model (20), which accounts for both steric and electrostatic effects. In the classical RSA model, when the interaction between a charged particle and an oppositely charged surface is very strong, the particle will maintain a fixed position at the surface and will not return to the bulk solution nor diffuse laterally into other areas of the surface. Particles will continue to randomly bind to the surface until no more particles fit on the surface without physical overlap, which for model planar disks would correspond to a maximum surface coverage corresponding to a fractional surface area of 0.55. In several experimental studies, it has been shown that for charged particles, the maximum fractional surface coverage is a function of solution ionic strength (20). At low salt concentrations, the maximum surface coverage is substantially less than the 55% predicted by the classical RSA model. However, this value has been found to increase with increasing conductivity approaching the expected limit asymptotically, suggesting that for charged particles bound to an oppositely charged surface, electrostatic repulsion must be considered. Applied to mAbs in ion-exchange resins, the modified RSA mechanism predicts that at low salt concentration, protein–protein repulsion forces molecules to bind strongly and adsorb at a distance from each other, leading to an overall low binding capacity. As the conductivity is increased, electrostatic screening will allow incoming protein molecules to attach to the surface at positions closer to bound molecules. In addition, the increase in conductivity will also weaken the protein–surface interaction and increase the lateral mobility of bound molecules that, while remaining bound, can now adjust their position on the surface, leading to an overall increase in the concentration of adsorbed protein. These effects are likely to complement the exclusion mechanism and to contribute to the increase in DBC with the initial increase in conductivity.

Although the exact mechanistic explanation of the intraparticle diffusive and adsorptive phenomena is of utmost importance, it is beyond the scope of this chapter. Instead, we focus on the practical application of such mechanistic insights to advances in process and technology development for industrial-scale protein purification.

#### 10.3.4 Factors Affecting the Critical Conductivity

Figure 10.3 shows that critical conductivity increases with decreasing pH. With isoelectric points of approximately 9, mAbs become increasingly positively



**FIGURE 10.3** DBC of a mAb as a function of pH and conductivity in SP XL. From reference 2, Copyright © 2006 by John Wiley & Sons. Reprinted by permission of John Wiley & Sons.

charged with decreasing pH. The increase in protein charge leads to stronger protein–resin binding and to stronger protein–protein electrostatic exclusion, resulting in hindered protein diffusion and lower DBCs. A higher ionic strength is thus required to shield the stronger electrostatic interactions caused by decreasing pH. Despite the apparent decrease in maximum DBC with decreasing pH, other experiments conducted at Genentech with multiple mAbs and cation exchangers have shown that the maximum achievable DBC at a given residence time remains approximately constant within the range pH 4.0–6.0.

Figure 10.3 clearly shows the combined effect of pH and conductivity in protein transport into the particle at a constant residence time. For example, at pH 4.0, the protein front migrates deeper into the particle with conductivity increasing from 1.2 to 25 mS/cm, and at a fixed conductivity of 10 mS/cm, protein transport into the core of the bead is greatly enhanced at pH 6.0 compared to pH 4.0.

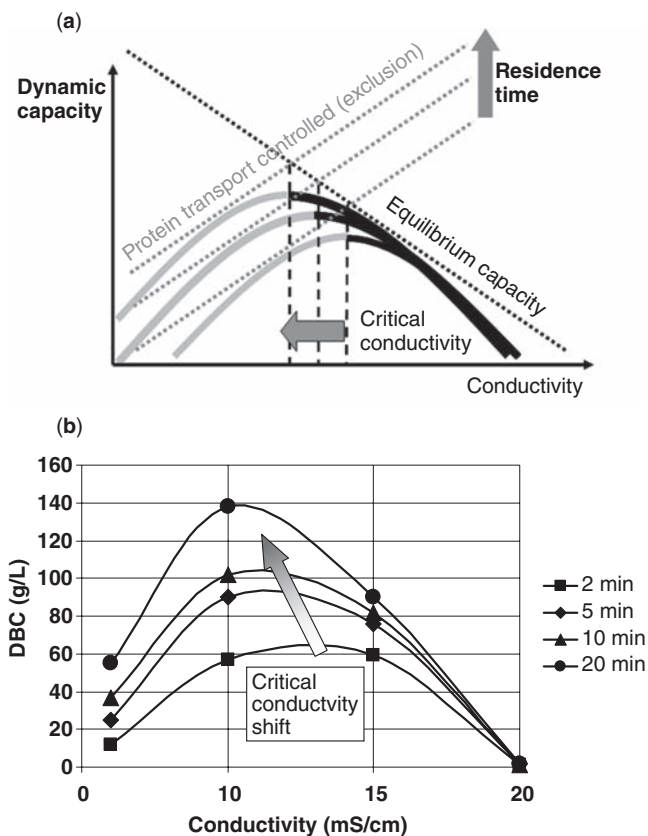
It has been shown (2) that there is a direct relationship between protein net charge and critical conductivity. In fact, a plot of critical conductivity versus protein charge for three different mAbs in two different resins condensed into a single and approximately linear trend (2). This may be in part due to the similarities between the two resins, SP FF and SP XL. They share the same agarose bead, SP-type charge groups, and charge density, differing primarily in the presence of a charged dextran polymer layer filling the pores in SP XL. Knowledge of this relationship is of crucial practical importance since it enables one to predict the optimal operating conductivity at any given experimental conditions for which protein charge is known.

It is common knowledge that under conditions where DBC is controlled by intraparticle protein transport, higher capacities can be obtained at longer load residence times, up to a point where the equilibrium capacity is reached. Perhaps not so obvious is an important prediction resulting from the trade-off

between the exclusion and equilibrium domains: at a given pH, the critical conductivity should decrease with increasing load residence time. At longer load residence times, optimal bead volume utilization can be achieved at a lower intraparticle protein transport rate. This enables operation at a lower conductivity, which in turn results in higher bound protein concentration and overall higher DBC.

This concept is illustrated in Fig. 10.4a. To test this hypothesis, we have investigated the effect of residence time on the DBC of a mAb in a high-capacity cation exchanger (21). Due to the limited number of experimental data points (Fig. 10.4b), it is not possible to identify the exact critical conductivity values, but the trend is clear, suggesting a decrease in the critical conductivity with increasing residence time.

Although the maximum DBC of a mAb has been shown to be relatively unaffected by resin ligand density within the range of 50–250  $\mu\text{M}$  (22), the



**FIGURE 10.4** Effect of residence time on critical conductivity. (a) Theory. (b) Experimental results with mAb on SP XL at pH 5.0. The arrow points in the direction of the shift in critical conductivity.



critical conductivity has been shown to increase with increasing ligand density. The authors postulated that stronger protein–surface interactions at higher ligand density cause further restrictions in intraparticle protein transport, which can be offset by a higher conductivity. Knowledge of the effect of ligand density on critical conductivity can be exploited for the design of novel cation exchangers for specific applications. For example, for the direct capture of mAbs from HCCF at 15 mS/cm, a threefold dilution would be required prior to loading onto typical CEX resins. At the 12,000 or 25,000 L bioreactor industrial scale, a threefold dilution prior to CEX would not be feasible due to tank volume and process time limitations. Nevertheless, a high-ligand-density cation exchanger could in principle be designed to enable a suitable combination of pH and critical conductivity that would not require dilution of the pH-adjusted HCCF.

### 10.3.5 Advances in mAb CEX Process Development

The notion of an intermediate critical conductivity for maximal DBC is of the greatest practical importance for industrial ion-exchange process development. It can no longer be assumed that the highest capacity will be obtained at the lowest ionic strength and pH. CEX process development must include studies of DBC as a function of load pH, conductivity, and residence time to identify the critical conductivity value for a given condition.

We have also shown that the critical conductivity is a function of the load residence time. Traditionally, optimization of load pH and conductivity has been conducted at a conveniently short load residence time to decrease the duration of scouting runs. The effect of load residence time on DBC has been independently determined at an arbitrary pH and conductivity. It is only near to the end of the process development activities that the load flow rate is typically adjusted according to mass throughput requirements and plant constraints. This leads to a suboptimal combination of load residence time, pH, and conductivity. Therefore, one must first identify the longest practical load residence time, from plant constraints and mass throughput requirements, and then conduct process development studies at the corresponding load flow rate.

Resin selection is typically done on the basis of required capacity and purity performance. However, the dependence of the critical conductivity on resin ligand density suggests that the selection of the resin should also take into consideration the conductivity of the pH-adjusted, undiluted upstream process pool and the critical conductivity at the same pH. As discussed earlier, for an upstream pool with high conductivity, a resin with a relatively high ligand density would require lower dilution or no dilution at all to condition the pH-adjusted load sample to the critical conductivity.

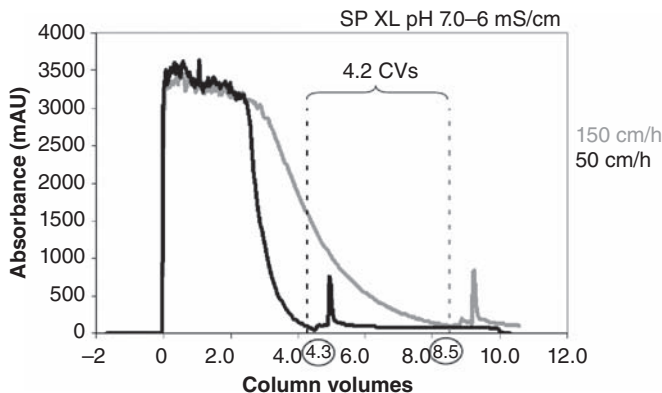
Neglecting the interdependence between load residence time, pH, conductivity, and resin ligand density leads to an underutilization of the CEX resin capacity and to suboptimal operating conditions. In the face of reported



advances in the understanding of mAb CEX phenomena, we propose a sequence for CEX process development activities below.

CEX process development should start with a clear definition of the step goals and operating constraints. This includes defining mass throughput and purity requirements, an operating window for product stability (pH, conductivity), upstream pool properties (pH, conductivity, hold stability, etc.) and manufacturing constraints such as available column sizes (diameter, bed height), buffer and pool tanks, flow rate capabilities, etc. With this information, one should be able to list a series of candidate resins that could potentially deliver the specified step goals within the identified constraints. For example, the cell culture titer and bioreactor volume may be such that capacities in excess of 80 g/L may be required to achieve the step goals and to enable facility fit. This would lead to the elimination of low-capacity resins. Depending on the number of remaining candidate resins, available time, and resources for process development including the availability of automated high-throughput screening equipment, it must be determined whether further resin elimination would be required at this point. If so, a preliminary purity performance assessment may be performed as part of the selection process. Purity is one of the most important step goals, along with yield. For a given resin, purity can be improved by tuning load conditions and by optimizing wash and elution phases. Nevertheless, the resin itself remains the most critical factor affecting pool purity, and a comparison of the purity performance of several candidate resins, even under suboptimized operating conditions, should still provide a fair qualitative ranking of the resins.

Once the list of candidate resins is reduced to a manageable number, one should then estimate the longest practical load residence time based on knowledge of plant and equipment constraints. Subsequent process development studies should be conducted at this load residence time until it is determined that a lower load residence time would still achieve the step mass throughput goals. Then, based on the knowledge of a suitable load operating pH range, the next step should be to determine DBC as a function of conductivity at least at three pH values corresponding to the extremes and the central point of the selected pH range. This will result in the determination of the critical conductivities as a function of pH and load residence time. The loading phase should be carried to at least 10% breakthrough. After a post-load wash with equilibration buffer, the column should be eluted at a standard reference condition, typically in a shallow conductivity gradient at a suitable pH (based on product stability, downstream load conditions, etc.). The elution of the DBC runs enables a preliminary assessment of the effect of the resin and of the loading conditions on purity performance and gives an initial idea of the target elution conditions for the product. After this set of runs, a single resin candidate along with a single pair of pH and conductivity values should be selected for subsequent testing, which should focus on the final optimization of post-load wash buffer conditions, elution strategy, phase durations, and phase flow rate.



**FIGURE 10.5** Effect of elution flow rate on pool volume for a mAb on SP XL.

The optimization of the elution phase requires a trade-off between resolution and pool volume. Shallow gradients provide the best purity performance but generate large pool volumes that may exceed the capacity of the pool tank, or may cause facility-fit problems downstream. Step elution, if feasible, is preferred over gradient elution for industrial applications. For a step elution strategy, elution flow rate can also be optimized to provide the smallest practical pool volume. Pool volume is ultimately controlled by the rate at which the mAb diffuses out of the resin bead, which is determined by the strength of the elution buffer and by the concentration gradient established between the intrabead mAb desorbed phase and the interbead solution. Therefore, the optimal elution rate for pool volume minimization will be only slightly faster than the rate at which protein diffuses out of the bead. Increasing the elution flow rate significantly beyond the minimum necessary to establish a concentration gradient to drive the protein out of the bead will only cause an unnecessarily large pool volume. Figure 10.5 shows an example of this strategy, where a reduction in the elution flow rate of a mAb in SP XL from 150 to 50 cm/h led to a 50% reduction in the pool volume.

## 10.4 HPTFF

### 10.4.1 Overview

HPTFF is an emerging purification tool that enables protein purification, concentration, and formulation in one unit operation (23–26). Whereas conventional neutral ultrafiltration processes (see Chapter 16) are limited to the separation of solutes with at least 10-fold difference in size, in one application of HPTFF, charged composite regenerated cellulose membranes are used for the separation of proteins, on the basis of size and charge, without constraints to their relative molecular weight. Several applications have been demonstrated including the separation of monomers from oligomers (24), an

antigen-binding fragment from a similar-sized impurity (27), protein variants differing at a single amino acid residue (28), and more recently using real-world industrial feedstreams, a 10-fold removal of *Escherichia coli* HCP (29), and a 100-fold reduction in Chinese hamster ovary host cell proteins (CHOP) (13).

High selectivity and throughput in HPTFF can be achieved by optimizing the operating configuration, membrane properties, and process variables. Unlike conventional membrane processes, HPTFF is operated in the pressure-dependent regime below the “transition point” in a plot of filtrate flux versus transmembrane pressure (23, 30, 31). This optimizes selectivity compared to that obtained in the pressure-dependent regime. The optimal selectivity and throughput occur at a specific filtrate flux. However, the formation of a pressure gradient along the length of the feed channel reduces the resolving power of conventional tangential flow filtration systems. The undesirable variation of the transmembrane pressure and flux can be overcome by establishing a cocurrent flow on the filtrate side of the membrane by using a recirculation pump to create a pressure gradient that balances the axial pressure drop in the retentate. In addition, the high permeability of HPTFF membranes commonly requires the use of a filtrate pump for precise control of the transmembrane flux.

Although HPTFF can be used in applications where the product of interest is recovered from the filtrate, in the purification of mAbs, the product is recovered from the retentate. Retention of the mAb is achieved by a combination of steric and electrostatic exclusion. With isoelectric points of around 9, mAbs have a net positive charge within a common operating pH range of 4.0–8.0. Therefore, for mAb applications, HPTFF membranes are positively charged. Selection of an appropriate membrane charge density and pore size distribution is crucial to achieve optimal selectivity and product yield. Typical molecular weight cutoffs for mAb purification range from 100–300 kDa. Higher ligand densities in general enable the use of higher conductivities while maintaining product retention and enhancing impurity removal.

The nature of buffer ionic species, pH, and ionic strength affect the selectivity and throughput of HPTFF processes through their effect on the molecular hydrodynamic volume. In general, higher conductivity shields protein charge with a consequent reduction in product yield and selectivity. A significant difference between the pH of the solution and the pI of the mAb will, in principle, reduce sieving and decrease purification performance by increased retention of impurities, but will enhance product yield as long as the product does not undergo any pH-induced conformational changes. Buffer pH values closer to the product pI tend to improve impurity clearance, but the enhanced sieving may have a negative effect on product yield. If the molecular weight and isoelectric points of the impurities are known, operating pH and conductivity may be selected to maximize selectivity. However, in the case of HCP, the impurities are usually not well characterized and the determination of optimal pH and conductivity must be carried out empirically.

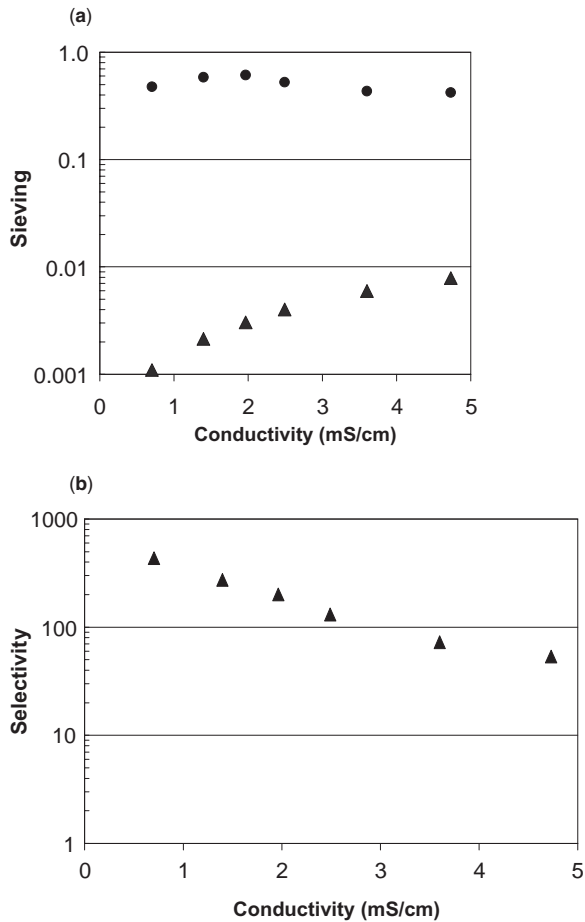
Optimization of HPTFF is accomplished by a trade-off between yield and purity, minimizing the membrane area within an acceptable process time. A

set of diagrams and equations has been developed (32) to enable analytical optimization of selectivity and mass throughput.

#### 10.4.2 Advances in HPTFF

As discussed above, improved performance in HPTFF can be obtained with cocurrent filtrate recirculation, which enables a near constant transmembrane pressure, and with a filtrate pump, which precisely controls the value of the transmembrane flux. It has been shown that mAb sieving can be reduced more than 10-fold by using cocurrent flow, while HCP sieving remains high (26). However, current large-scale ultrafiltration systems in the biotechnology industry do not have the option of operating with cocurrent flow and would require a significant modification to accommodate the cocurrent flow loop, along with additional pumps and process control capabilities. This would become a major barrier to the implementation of HPTFF in industrial mAb purification. Thus, elimination of the requirement for cocurrent filtrate flow is instrumental for the widespread use of HPTFF. Zydney and van Reis (25) suggested that in theory, using a membrane with lower permeability may enable operation without a cocurrent filtrate loop. Decreasing the membrane permeability reduces the relative difference between the inlet and outlet transmembrane pressure. As a result, the transmembrane flux should become more homogenous, and at a sufficiently low value of membrane permeability, the performance should approach that of the cocurrent flow configuration. Further improvement in the performance without cocurrent flow could also in principle be obtained by other means of reducing the pressure drop through the HPTFF module, for example, by reducing the feed flow rate or by using an open channel configuration (25).

The authors estimated that a two- to threefold reduction in membrane permeability would be required for the performance without cocurrent flow to approach that with cocurrent flow. The challenge was to determine how to decrease the membrane permeability without affecting the sieving characteristics dictated by the charge density and pore size distribution. That was achieved through a collaborative effort between Genentech and Millipore Corporation. The next step was to determine the target membrane pore size and charge density values that, in conjunction with the reduced hydraulic permeability, would provide optimal performance under industrially relevant conditions. A new series of membranes with lower permeability, and varying pore sizes of 100–300 kDa, was charged to a relatively wide range of charge densities and were tested for mAb yield and selectivity. In the first series of tests, the membranes were evaluated with a chemically defined feedstock consisting of a mAb and three additional proteins: two Fab'2 and one Fab fragment (33). In contrast to the order of magnitude difference in mAb sieving previously observed with and without cocurrent flow (26), mAb sieving was comparable for these configurations. In general, sieving was still slightly lower and the selectivity slightly higher for operation with cocurrent flow. Nevertheless,



**FIGURE 10.6** HPTFF performance with real-world CHOP feedstock without cocurrent flow. (a) mAb and CHOP sieving versus conductivity. Triangles: mAb sieving; circles: CHOP sieving. (b) CHOP selectivity versus conductivity.

the reduction of membrane permeability enabled a significant increase in the relative performance without cocurrent flow. The best performing device was further evaluated with a real-world CHOP feedstock (Fig. 10.6). For the first time, CHOP selectivity greater than 100 was observed without cocurrent flow under industrially relevant conditions.

### 10.5 A NEW NONAFFINITY PLATFORM

As discussed in Section 10.2, there is a cost pressure to eliminate the Protein A step and to further simplify the current mAb purification platform. Others

have succeeded in demonstrating the feasibility of nonaffinity processes (see Chapter 5). Fahrner and colleagues (12) integrated different conventional chromatography steps into a three-column nonaffinity process capable of delivering comparable yield and product quality to that of the Protein A-based process. More recently, Arunakumari and colleagues (34) developed a two-step process based on a CEX capture column followed by AEX flow-through membrane chromatography, which resulted in greater than 80% process yield, higher batch capacity, and shorter process time.

The success of two-step nonaffinity approaches in mAb purification depends on the stringency of final product quality requirements and the sensitivity of the analytical assays. The recent advances presented above, particularly the elimination of the cocurrent flow requirement for HPTFF, which enables the simple replacement of neutral ultrafiltration membranes by HPTFF devices and the use of the entire installed base of ultrafiltration systems, have enabled the industrial feasibility of a new two-chromatography step nonaffinity approach for the industrial-scale purification of mAbs. This new nonaffinity process uses CEX as a capture step, followed by AEX membrane chromatography as a polishing step, followed by HPTFF.

A recent study at Genentech (13) has demonstrated the two-chromatography step nonaffinity process at pilot scale under industrially relevant conditions with process yield and product quality comparable to those obtained with the conventional affinity process. This new process, built from recent advances in technology and process development, represents a step forward toward process simplification and cost efficiency, crucial to address the challenges posed to the biotechnology industry, and establishes a reference for a new platform approach to industrial mAb purification.

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## CONTINUOUS CHROMATOGRAPHY FOR THE PURIFICATION OF MONOCLONAL ANTIBODIES

THOMAS MÜLLER-SPÄTH AND MASSIMO MORBIDELLI

### 11.1 INTRODUCTION

Biomolecules such as therapeutic proteins and peptides are taking an increasing share of the pharmaceuticals market. By November 2005, nearly 30 monoclonal antibodies (mAbs) had been approved for therapeutic use or as *in vivo* diagnostics, and approximately 150 more were in clinical development (1). With steady improvements in expression technology, the yield of mAbs from the upstream part of the production process has increased dramatically.

Since mAbs are generally produced by fermentation, they are accompanied by large amounts of impurities such as host cell protein (HCP), DNA, and media components. Depending on the fermentation conditions, degraded, truncated, and aggregated mAbs may also be found in the cell culture supernatant (2). In addition, the product itself may consist of different variants. For example, microheterogeneity caused by deamidation has been reported (3), and different glycan chain structures are very common for mAbs and other therapeutic proteins such as erythropoietin (EPO) (4). It is known that glycosylation affects the biological activity and pharmacokinetics of such molecules significantly (4). Affinity chromatography, such as Protein A chromatography in the case of mAb purification, does not distinguish among the variants and glycoforms (see Chapter 4). This stresses the importance of product specification—the product may be defined as a single variant, e.g., the one with the highest biological activity, or as a mixture of variants that may have a lower

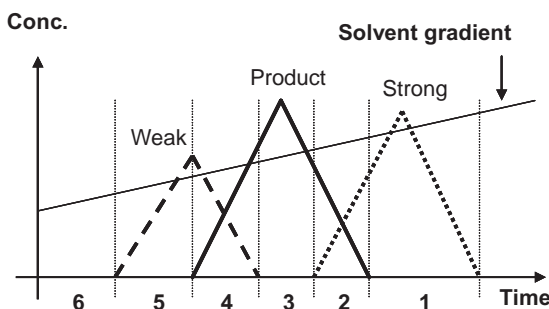
overall activity. In summary, the purification of mAbs can be regarded as the separation of a mixture from a more complex mixture.

The screening results for suitable buffer and adsorbent material combinations depend strongly on the properties of the product and therefore are not discussed here. The scope of this chapter is to outline changes in the process in order to improve separation, and to focus on continuous chromatography in particular.

## 11.2 PRODUCT VARIANTS AND THE SEPARATION PROBLEM

When chromatographic purification is carried out in batch gradient mode, e.g., the capture of a mAb from cell culture supernatant, the product elutes from the adsorbent as a peak following a change in the eluent composition. The product is flanked by weakly adsorbing, early-eluting impurities and by strongly adsorbing, late-eluting impurities. A three-fraction separation is required to obtain the product that elutes in the center, as illustrated in Fig. 11.1. The chromatogram indicates two main limitations of the separation: first, the early- and late-eluting product fractions contain a large number of impurities, and the overlapping regions of product and impurities may not be in specification. Second, impurities eluting in between the variants of the product may not be separated out without reducing the yield of the desired product.

In order to obtain a high yield despite these limitations, either the experimental conditions need to be changed, i.e., the type of adsorbent or buffer, or another process should be chosen. An example of the former approach is the use of affinity materials that separate the variants using adsorption between highly selective ligands and regions common to all product variants. For the purification of mAbs, Protein A chromatography makes use of this concept by exploiting the interaction between the Protein A ligand and the fragment-crystallizable (Fc) region on the mAb molecule. The ability of Protein A to



**FIGURE 11.1** Solvent gradient chromatography: scheme of a three-fraction separation with weakly adsorbing, early-eluting impurities, product, and strongly adsorbing, late-eluting impurities. The numbers below the time axis denote the sections of the chromatogram.

bind IgGs of subclasses 1, 2, and 4 but not subclass 3 demonstrates the strong selectivity of this ligand. Affinity chromatography essentially changes the purification problem from a three-fraction to a two-fraction separation and is able to clear impurities that elute in between the product variants in, e.g., ion-exchange (IEX) chromatography or hydrophobic interaction chromatography (HIC).

### 11.3 DEFINITION OF PERFORMANCE PARAMETERS

Before discussing chromatography methods in detail, we need to define the performance parameters used to judge the quality of chromatographic separations. The most important performance parameters are yield, purity, productivity, and eluent consumption. The yield is defined as the ratio between the mass of the target compound in the product fraction and the mass of the target compound that entered the column during the loading phase. The purity is presented either as a percentage, based on the peak areas in an analytical chromatogram, or by the ratio of impurity and product in the product fraction, e.g., in nanogram HCP per milligram mAb or parts per million. Productivity is defined as the mass of material produced per resin volume and process time. Productivity is also equivalent to the product of load (mass of material loaded per resin volume and process time) and yield.

Selectivity is a parameter that depends on the stationary phase and buffer system. It is a relative parameter that is defined as the ratio of the retention factors of two components. If the selectivity of component A with respect to component B is  $<1$ , component A elutes earlier than component B. If it is  $>1$ , component A elutes later than component B.

### 11.4 GRADIENT CHROMATOGRAPHY FOR BIOMOLECULES

Assuming the choice of a certain stationary phase and buffer system has already been made, the simplest purification approach is single-column batch chromatography using a step gradient elution. Given the large number of impurities in biochromatography, a step gradient certainly will not achieve a pure product, since most components that have bound to the adsorbent are eluted at the same time and the product is highly impure. In addition, step gradients involve the greatest possible perturbations in mobile phase composition and the accompanying effects are not necessarily beneficial. For instance, in weak cation-exchange (CEX) chromatography, a positive salt gradient leads to a drop in pH (5). Multistep gradients rarely improve the situation, since many impurities have selectivities close to 1 with respect to the product, meaning very similar adsorptive properties.

Starting from batch chromatography, the most effective parameter for process improvement is the solvent gradient, e.g., a salt or pH gradient in IEX

chromatography. Linear solvent gradients facilitate separation due to the gradual change in the eluent concentration. The shallower the gradient, the greater the product purity. However, depending on the selectivities of the impurities, some of the product fractions may still fall outside specification. Another drawback of shallow gradients is the decrease in productivity and the increasing product dilution. Mathematical modeling is a valuable tool that can be used to find the optimal operating conditions in terms of yield, purity, and productivity.

## **11.5 CONTINUOUS CHROMATOGRAPHY TO INCREASE PRODUCTIVITY**

Productivity is an issue for batch gradient chromatography in general, not only in processes employing a shallow solvent gradient. In particular, each part of the column remains unused after the peak passes through, until the next injection.

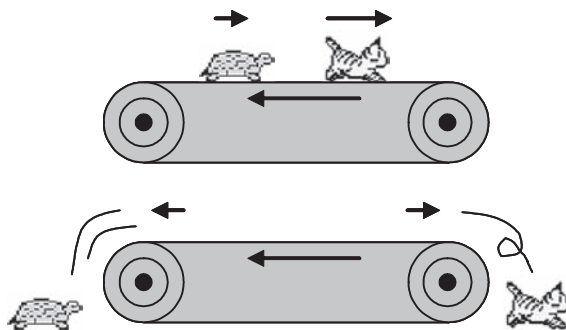
Continuous chromatography offers a way to increase productivity as the column is loaded continuously. Through accumulation of product, resin utilization and hence productivity can be increased significantly. If, in addition, countercurrent movement of the stationary and mobile phases is achieved, the separation efficiency increases, allowing the removal of high levels of impurities. Consequently, a partial separation of the components is sufficient to obtain a highly pure product.

### **11.5.1 The Concept of Continuous Chromatography**

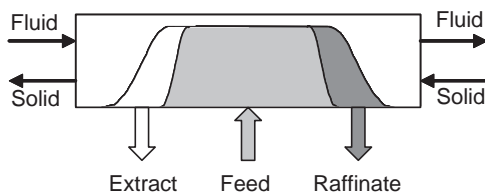
A simple allegory can be used to explain the concept of continuous chromatography: a turtle and a cat, representing two components in a mixture, are dropped on a conveyor belt and immediately start to run to the right. The cat runs faster than the turtle, representing the weaker adsorbing species (Fig. 11.2). The conveyor belt moves to the left, opposite to the direction in which they are running (countercurrent movement). In continuous chromatography, the conveyor belt corresponds to the moving stationary phase. Initially, the speed of the conveyor belt is slow, so both animals reach the right-hand side. However, if the speed of the conveyor belt is increased above a certain value, the turtle will no longer reach the right-hand side but will instead be transported to the left. The cat still reaches the right-hand end side and the animals are thus separated successfully. This concept works also when turtles and cats are dropped continuously on the conveyor belt.

### **11.5.2 The Simulated Moving Bed (SMB) Process**

A setup where the stationary phase moves continuously, a so-called true moving bed (TMB), is hard to realize from an engineering point of view. This



**FIGURE 11.2** Concept of continuous chromatography.

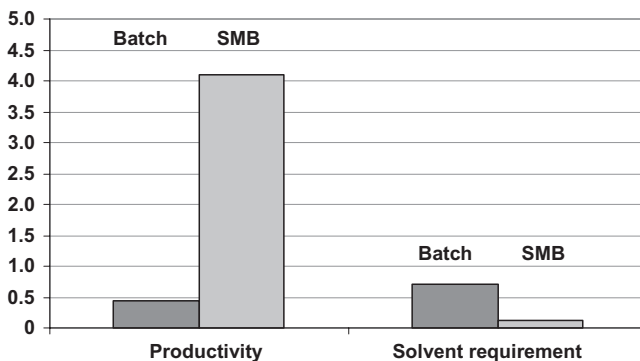


**FIGURE 11.3** Partial separation of two components in continuous chromatography.

has led to the development of the SMB technology, where valves are switched in such way that moving the inlet and outlet ports simulates the movement of the adsorbents and the stationary phase. An SMB has four sections devoted respectively to the adsorption of component B, the adsorption of component A, the desorption of component B, and the desorption of component A. The design of SMB processes has been described for linear and Langmuir adsorption isotherm systems (6). In SMB, due to transport effects, there exists a section in the vicinity of the feed inlet where the two components are mixed, but they also form regions where they have been separated and can be extracted in pure form. This concept is illustrated in Figs. 11.2 and 11.3 and shows that partial separation of the components is sufficient to obtain high purity.

### 11.5.3 Advantages and Disadvantages of Batch and SMB Chromatography

As mentioned above, one advantage of SMB over batch chromatography is that it requires only a partial separation of the components in order to obtain a product of high purity and yield. In batch chromatography, baseline separation is required to achieve high purity, which in turn reduces yields and productivity. Due to the accumulation of the products in the SMB unit, the resin utilization is improved compared to batch chromatography. The feed is loaded



**FIGURE 11.4** Comparison of batch high performance liquid chromatography (HPLC) and SMB purification of a pharmaceutical intermediate racemate mixture on a chiral HPLC stationary phase. Productivity is given in kilogram racemate per kilogram of chiral stationary phase per day; solvent requirement is given in solvent per gram racemate. Adapted from reference 8.

continuously so the productivity is high and the solvent consumption is reduced. The disadvantages of SMB include its inability to utilize gradients other than step gradients and its limitation to two-fraction separations. As explained earlier, in most biochromatography separation problems, at least three-fraction separations are required and solvent gradients are advantageous.

The original SMB process developed in the 1950s was used for the separation of small molecules (7). It is particularly suited for difficult separations where the components have a selectivity close to 1 (such as the separation of enantiomers). In such cases, the separation into two fractions exactly fits the SMB concept, and SMB clearly outperforms batch chromatography in this field (8). Figure 11.4 shows data concerning the purification of enantiomers from a racemic mixture of pharmaceutical intermediates. The solvent consumption per mass of product is significantly lower for SMB, while the productivity is higher.

As a consequence, there are currently no industrial SMB processes applied in the purification of complex biomolecules. As described above, the use of affinity chromatography transforms the separation problem from a three-fraction into a two-fraction separation, theoretically allowing the use of SMB. However, the selectivities of the components to be separated with the affinity material are in general so large that this kind of process has no advantage compared to batch affinity chromatography in terms of purity and yield, as it uses the same stationary phase (9). Only in rare cases, when one of the components is very easily separated (baseline separation) from the others (10), can SMB be used for three-fraction separations. If no baseline separation is present, an SMB cascade (11) that is not economically viable is required.

In contrast, batch chromatography has a very successful track record in biochromatography, since it can be run in gradient mode with the concentration of a modifying substance, such as NaCl, changing over time. Multifraction

**TABLE 11.1 Advantages and Disadvantages of Batch and Continuous Chromatography**

Technology	Batch	SMB	MCSGP
Advantage	Three-fraction separation Eluent gradients Only one large column	High efficiency Continuous feed Countercurrent operation	High efficiency Continuous feed Three-fraction separation Eluent gradients Countercurrent operation
Disadvantage	Low efficiency Discontinuous feed	No three-fraction separation Only step gradients At least four small columns	At least three small columns

separations are therefore possible. Disadvantages include low productivity, low stationary phase utilization, and high solvent consumption. These disadvantages of batch and SMB processes have led to the development of a new process: multicolumn countercurrent solvent gradient purification (MCSGP), which combines the advantages of batch chromatography and SMB.

An overview of the advantages and disadvantages of the three technologies is provided in Table 11.1.

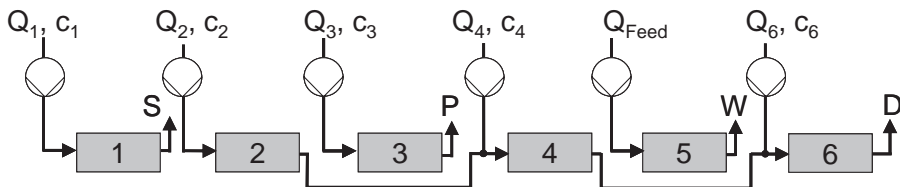
## 11.6 THE MCSGP PROCESS

The MCSGP process is a hybrid process between batch and continuous chromatography, combining the advantages of batch chromatography (principally the ability to perform three-fraction separations with solvent gradients) with the advantages of SMB (i.e., the high efficiency and low solvent consumption) (12, 13, 14). The MCSGP process may be used for capture and polishing applications. MCSGP uses conventional chromatographic stationary phases such as IEX, HIC, and reversed-phase resins, as well as conventional columns, valves, and piping. The use of expensive affinity material is avoided. The degree of complexity is comparable to that of SMB.

Historically, the first application was for the separation of peptides in reversed-phase mode (15), which lies outside the scope of this book. We will focus here on the use of MCSGP to capture mAbs from clarified cell culture supernatants and the separation of mAb variants, both using IEX stationary phases.

### 11.6.1 MCSGP Process Design

As is the case for SMB, the MCSGP process can be divided into different sections, with tasks defined according to the batch chromatogram in Fig. 11.1. The



**FIGURE 11.5** Principle of the MCSGP process.  $Q_i$  and  $c_i$  denote the flow rates and solvent concentration entering the single sections.

basic principle of the process is shown in Fig. 11.5. In the first section, the strongly adsorbing impurity S is eluted. In the second section, the overlapping part between S and the product P is recycled internally to the fourth section. In the third section, the product is eluted. In the fourth section, the overlapping part between P and weakly adsorbing impurities W is recycled to the sixth section, while in the fifth section, the weakly adsorbing impurities are eluted and the product is fed into the sixth section, where the recycled P is adsorbed. Only weakly adsorbing impurities leave the system through the desorbent outlet D. The columns are switched from high to low section numbers.

The process can be operated e.g., in a continuous six-column mode (15) or in a three-column semicontinuous mode (16) where the tasks of recycling and elution alternate. The operating parameters, i.e., the six flow rates and the six gradients, can be derived from the batch chromatogram. For more details on the process design, the reader is referred to the literature (15, 16). Depending on the process design, the MCSGP process requires a certain time to reach a cyclical steady state. However, because of the internal recycling, no product is lost during the start-up phase.

### 11.6.2 MCSGP for the Capture of mAbs from Clarified Cell Culture Supernatants

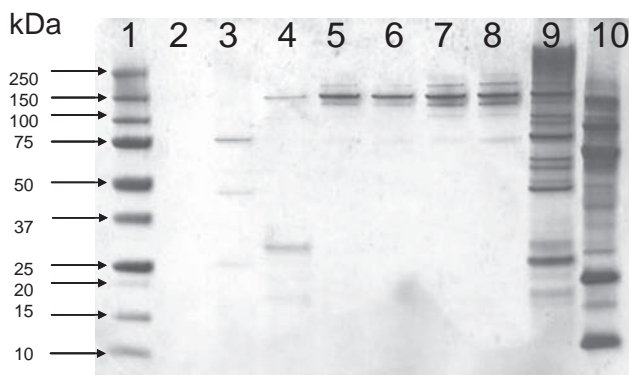
At this point in time, MCSGP has been applied successfully in the purification of mAbs from high- and low-titer clarified cell culture supernatants using IEX material. Because of confidentiality issues, only data concerning a low-titer Chinese Hamster Ovary (CHO) cell culture supernatant ( $c_{\text{mAb}} = 0.08 \text{ g/L}$ ) can be presented here. The purity of the supernatant was 235,000 ng HCP/mg mAb. Gel electrophoresis and size exclusion chromatography were used to provide a qualitative indication of purity, while mAb yield and purity were established quantitatively using an analytical Protein A method and a CHO cell enzyme-linked immuno sorbent assay (ELISA), respectively. The qualitative fractionation of a batch gradient eluate showed that the mAb was accompanied by a large number of impurities, some early and some late eluting, requiring a three-fraction separation of the mixture. In addition, the presence of charged product variants was demonstrated by isoelectric focusing. One major variant could be resolved using analytical CEX material. Both the batch gradient and



MCSGP purifications were carried out using preparative CEX material (Frac-togel SE HiCap, Merck, Germany).

The process design was based on a batch gradient experiment. Two process designs were chosen. The first aimed to maximize the yield of all mAb variants, while the second sought to purify the main variant only. Where the high overall yield was desired, different variants were captured along with impurities eluting between them. While the overall yield was 93% (and 94% for the main variant), the product purity was 5100 ppm HCP, corresponding to an HCP clearance of approximately 50-fold. In the second design, the overall yield was 81% (and 98% for the main variant). The purity in this case was 2000 ppm, corresponding to an HCP clearance of approximately 120-fold. This example shows the supreme importance of product definition. If the product comprises the main mAb variant alone, e.g., due to its biological activity, the second process design would clearly be preferred. As a comparison to the MCSGP runs, batch chromatography was carried out using the same stationary phase. The overall yields of the batch CEX runs were approximately 80%, with purities of approximately 19,000 ppm corresponding to a clearance of 13-fold. Affinity chromatography using Protein A (MabSelect Sure, GE Healthcare, Uppsala, Sweden) had an overall yield of 82% and an HCP clearance of 340-fold. The solvent consumption results were 63 mL/mg mAb for the batch process, 43 mL/mg mAb for the continuous process, and 20 mL/mg for Protein A affinity chromatography.

The results of the qualitative purity assays are shown in Fig. 11.6, in the form of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Compared to the unprocessed supernatant (lane 9), batch IEX



**FIGURE 11.6** SDS-PAGE, silver-stained gel: lane 1, marker; lane 2, desorbent outlet of MCSGP unit; lane 3, MCSGP, weakly adsorbing impurity outlet; lane 4, MCSGP, strongly adsorbing impurity outlet; lane 5, MCSGP product; lane 6, MCSGP product; lane 7, Protein A-purified product; lane 8, batch CEX-purified product; lane 9, clarified cell culture supernatant; lane 10, marker.

(lane 8) and Protein A chromatography (lane 7) and MCSGP (lane 5) all clear the majority of the impurities. Although no conclusions can be drawn as to which process is preferable (an ELISA is required for quantitation), the gel shows that the continuous three-fraction separation by MCSGP was successful, since different contaminants were found in the outlets for weakly adsorbing (lane 3) and strongly adsorbing (lane 4) impurities.

No attempt was made to optimize the productivity nor the eluent composition of the MCSGP process since the mAb concentration in the supernatant was very low. This justifies why, in this case, the productivity of the IEX batch process was higher than that of the MCSGP process, which is due to the fact that the MCSGP process uses four columns. However, our results indicate that lowering the productivity, e.g., by using a shallower gradient, does not significantly influence the purity in this specific case.

When comparing the batch and MCSGP processes, it is clear that MCSGP allows the production of mAbs whose purity is 10-fold greater than mAbs produced by batch IEX chromatography at a comparable yield. In general, MCSGP generates material whose purity is higher than the purest fraction from a corresponding batch eluate. This is due to the internal recycling, which increases purity, prevents product losses, and results in further separation of the product and the impurities. In theory, if displacement effects are present between the product and the impurities, the purity is improved even further. Such effects have been exploited successfully for peptide purification (15), but have yet to be observed with mAbs. When comparing Protein A chromatography and MCSGP, it becomes clear that in this case, the affinity chromatography method results in a threefold purer product at a comparable yield. The performance of the MCSGP process is also strongly dependent on the distribution of impurities and product variants with respect to their adsorptive properties.

### 11.6.3 MCSGP for the Separation of mAb Variants

The purification of three mAbs differing in the number of C-terminal lysine groups represents a typical polishing application—the product, the intermediate fraction of a gradient elution, was clearly one of the major constituents of the feedstock. We used a preparative weak CEX resin (Fractogel COO-(S), Merck) for purification in batch mode. Even when very shallow salt gradients were used and the product was eluted in more than 50 column volumes (CV) of buffer, it was not possible to obtain the intermediate variant with a yield greater than 10% at a purity greater than 80%. The challenge was to purify the intermediate variant to >90% with a yield also >90%. The productivity was set low at process design due to the limited amount of feedstock available and was not considered a key performance parameter in this case.

This separation problem could not be solved by batch affinity chromatography since Protein A is not able to distinguish among mAb variants. With the MCSGP process in place, it was possible to obtain the product with both

yield and purity at 93% (17). The separation problem and the result of the purification by MCSGP are shown in an analytical chromatogram in Fig. 11.7.

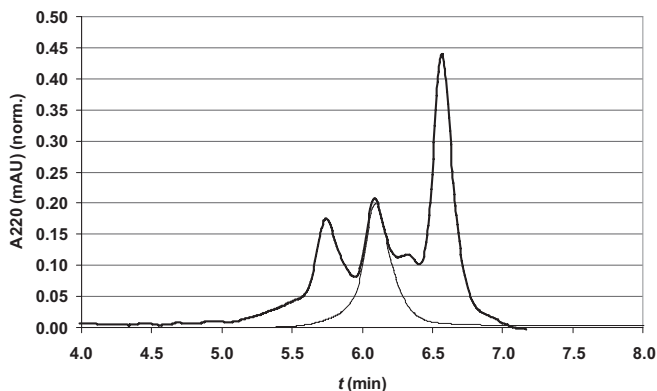
This example shows that, disregarding the productivity, the MCSGP process is able to operate in yield/purity regions that are inaccessible to batch processes. This advantage is particularly valuable in polishing steps where some value has been added to the product during the previous steps and product loss cannot be tolerated.

### **11.7 UPGRADES FOR CONTINUOUS PROCESSES TO IMPROVE STABILITY**

When dealing with crude feedstocks such as cell culture supernatants, the columns are subject to fouling. In continuous processes, irreversibly adsorbing impurities may accumulate in the unit, leading to higher pressure drops and reduced product purity. In such cases, the introduction of a cleaning-in-place (CIP) step into the process is imperative. By adding another section to the process, it is possible to use harsh conditions (e.g., cleaning with sodium hydroxide) without affecting the separation process. In addition, if feed conditioning such as dilution is required but the conditioned feed is unstable, inline mixing may be applied. Both process upgrades have been implemented for the purification of mAb from clarified cell culture supernatants, and the process was operated successfully for 6 days in a row (18). The compatibility of continuous processes with good manufacturing practice (GMP) guidelines has been demonstrated in various cases, e.g., for the separation of chiral components by SMB.

### **11.8 IMPACT OF INCREASING FERMENTATION TITERS**

The following considerations outline the impact of increasing fermentation titers on the capture step of downstream processing and are in principle valid for all continuous processes where loading and nonloading tasks are made in parallel. For the capture step, productivity is the key performance parameter. As shown earlier the load and also the productivity are proportional to the product titer. If we now consider a given fermentation batch with a fixed volume of supernatant to be loaded onto the column, we see that the larger the titer, the shorter the loading time of the column becomes in proportion to the total processing time, which includes washing, elution, CIP and re-equilibration, since the capacity of the stationary phase is fixed. Therefore, with increasing titers, column loading is no longer the bottleneck, but the process time is dominated by nonloading tasks. On the other hand, a larger capacity of the stationary phase leads to a longer loading time in relation to the nonloading tasks. In batch chromatography, all tasks are performed consecutively, while in a continuous process, they run simultaneously with the loading. As

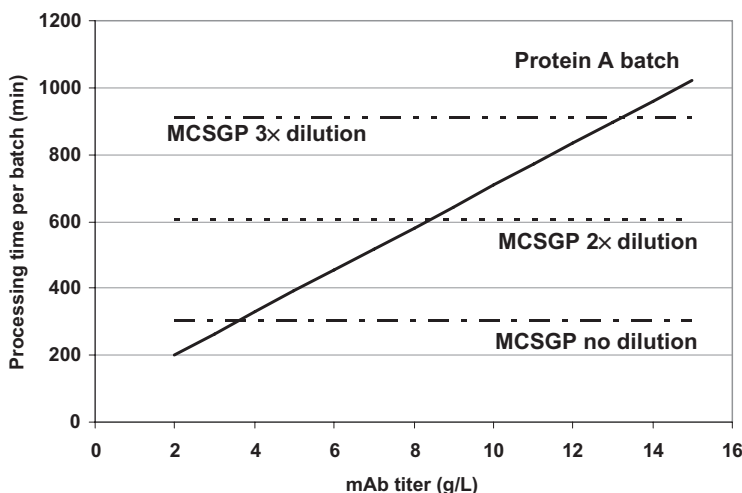


**FIGURE 11.7** mAb variant polishing. Analytical CEX chromatogram of three-variant feed mixture and MCSGP product fraction.

long as the loading column of the continuous process is operated below its capacity, which can be ensured by setting the switch time appropriately, the unit can be loaded continuously.

The consequence is shown in Fig. 11.8, where the processing time needed to capture mAb from the supernatant is shown as a function of the fermentation titer for IEX MCSGP and Protein A affinity chromatography. For the continuous process with the given switch time, no product breakthrough is expected during the loading step, so the processing time is independent of the titer. In contrast, for batch chromatography where the nonloading tasks dominate the process, the total processing time increases in a linear fashion as the titer increases. More cycles are required to process each batch. As the loading time decreases with respect to the nonloading tasks, the simultaneous operation of the latter improves the productivity.

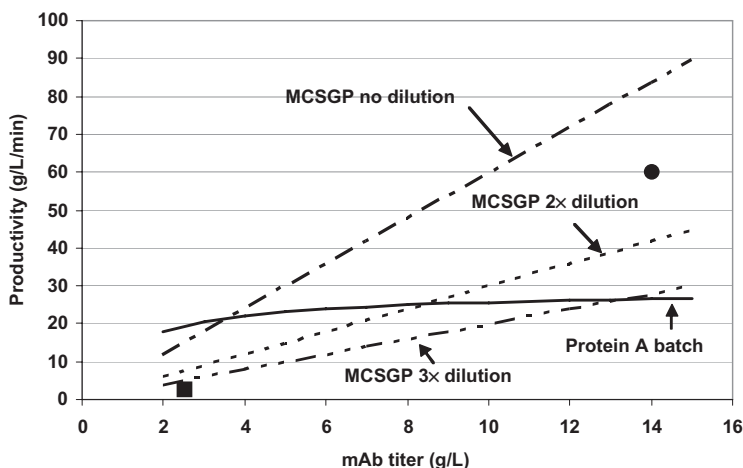
In Fig. 11.8 it is shown that for small titers, batch affinity chromatography is advantageous with respect to the overall processing time per fermentation batch, while for large titers, continuous IEX chromatography is better. The break-even point between the two processes depends strongly on the feed dilution required to shift the supernatant to binding conditions suitable for the IEX continuous process. If a threefold dilution is required, this value is approximately 13 g/L, while for zero dilution it is at 3.5 g/L. Fig. 11.9 shows the corresponding productivities for the batch affinity process and the continuous process according to the fermentation titer. As the fermentation titer increases, the break-even points shown in Fig. 11.8 also become clear. It was assumed in both cases that there is a 95% yield, which is realistic for the continuous process due to the internal recycling. The impact of dilution highlights the importance of feed conditioning on one hand and of stationary phase screening on the other. The manufacturers of stationary phases now aim to develop non-affinity-based materials that require minimal feed conditioning and dilu-



**FIGURE 11.8** Processing time per fermentation batch (20,000 L) as a function of the mAb titer. Further assumptions (Protein A column: 200-cm deep, 20-cm high, load 30 g/L, flow rate 0.42 CV/min, wash 10 CV, elute 5 CV, equilibrate 10 CV, yield 95%; MCSGP: four columns but same total resin volume and bed height as Protein A process, same linear loading flow rate, maximum load 84 g/L for  $c_{\text{mAb}} = 15$  g/L, undiluted, switch time 10 min, yield 95%).

tion while at the same time providing high capacity. A second trend is the development of high-throughput materials that improve the productivity. Also, a trend in cell line development can be observed toward the engineering of mAbs with a high isoelectric point that strongly adsorb on CEX materials without supernatant conditioning. Taken together, Figs. 11.8 and 11.9 show that the advantages of continuous chromatography over batch chromatography increase with fermentation titers. Fig. 11.9 shows an experimental point obtained using a supernatant with a mAb concentration of 2.5 g/L, diluted 1:4 with water and an experimental point using an ultrafiltrated, nondiluted supernatant with a mAb concentration of 14 g/L. A good agreement is observed between experiments and theoretical predictions. Our latest results confirm experimentally that IEX-MCSGP with a high-throughput material outperforms affinity chromatography in terms of productivity even if feed dilution is required and the mAb titer of the cell culture supernatant is in the range of 2 g/L.

As discussed above, increasing product titers require additional cycles in a batch process, in turn demanding more frequent replacement of the column packing. Alternatively, in order to process the same volume of feedstock in the same time, more stationary phase can be used. As a consequence, if Protein A affinity material is used, the cost for stationary-phase material ends up accounting for two-thirds of the total downstream costs as mAb titers reach



**FIGURE 11.9** Productivity as a function of the mAb titer. Lines and assumptions as described Fig. 11.8. Black symbols represent experimentally confirmed point for low- (square) and high- (circle) titer supernatants.

10 g/L (19). At the same time, the overall downstream costs increase. Conventional IEX materials combined with continuous chromatography are a cost-effective option here. In polishing applications, the issue of feed conditioning is of minimal importance since the adsorptive properties of the product and impurities are the major performance-influencing parameters.

## 11.9 OUTLOOK

Protein A affinity chromatography remains the workhorse for the capture of mAbs from complex feedstreams because it is a straightforward and versatile process that generally delivers a very pure product (see Chapter 4). However, with increasing product titers, the costs of Protein A affinity chromatography are becoming prohibitive and equally or more productive process alternatives are required, which are particularly suited for high-titer supernatants. In the midterm, continuous chromatography using cost-effective stationary phases may become more and more important, especially since this outperforms batch nonaffinity chromatography. Such continuous processes need to account for the complexity of bioseparations by incorporating linear solvent gradients and by achieving three-fraction separations, which is possible using the MCSGP process. MCSGP may also be used to capture mAb fragments and dedicated mAb variants that cannot be purified with Protein A chromatography. In such cases, IEX-based continuous chromatography is an excellent option for capture and polishing applications.

## 11.10 ACKNOWLEDGMENTS

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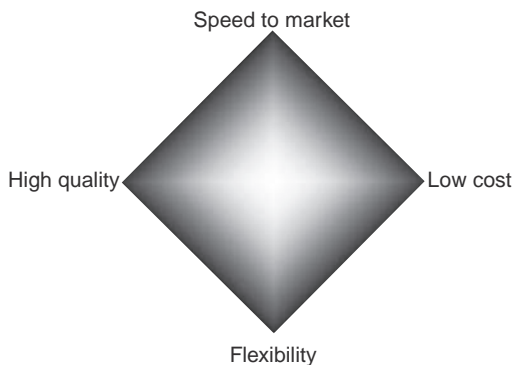
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## PROCESS ECONOMIC DRIVERS IN INDUSTRIAL MONOCLONAL ANTIBODY MANUFACTURE

SUZANNE S. FARID

### 12.1 INTRODUCTION

The success of therapeutic monoclonal antibodies (mAbs) in the treatment of indications such as cancer and autoimmune diseases has fuelled clinical trial activities, and in recent years, mAbs have become the fastest-growing segment of the biopharmaceutical industry (1, 2). Historically, attractive returns coupled with potential losses in revenue resulting from delays in product approval have made companies focus on time to market rather than on improving process economics (3–5). However, mAbs are among the most expensive drugs—the annual cost per patient can reach \$35,000 for antibodies treating cancer. With intensified competition on the way (6) and increasing pressure from healthcare providers, ultimate success for the next generation of mAb therapeutics will depend on economic factors. Hence, as the mAb sector has matured, emphasis has turned to all four main sources of competitive advantage: time to market, high quality, flexibility and low costs, and the motto “fail fast, fail cheap” (Fig. 12.1). As a result, production costs and capacity utilization are becoming critical success factors for the industry (8, 9). Continuous improvements in platform technologies are being sought so as to keep manufacturing off the critical path. The high demand for mAbs initially encouraged the search for alternative expression systems with higher productivities. However, increasing titers in mammalian cell cultures has recently shifted the focus of process develop-



**FIGURE 12.1** Main sources of competitive advantage in biotechnology development (extended from reference 7). Emphasis is placed on the motto “fail fast, fail cheap,” but these goals are often in conflict with one another and need to be reconciled to achieve the optimal strategy.

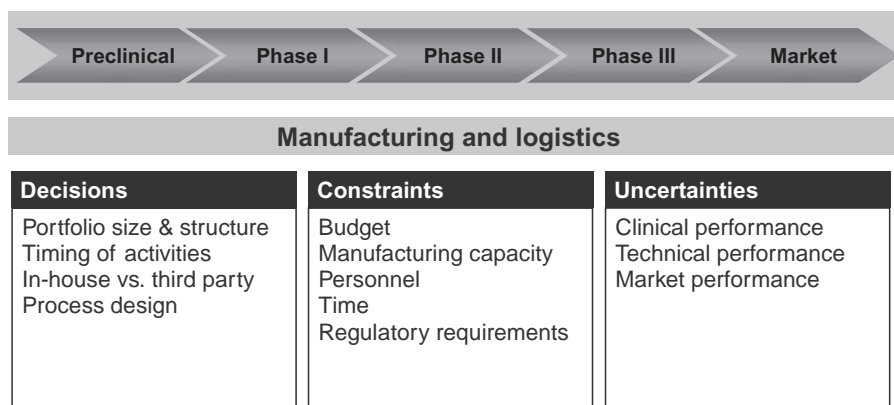
ment efforts toward improving the economics of product recovery and purification. Some argue that a paradigm shift may be required to ensure timely and cost-effective delivery of future mAb candidates. Searching for strategies that drive down costs while improving operational efficiencies is complex since this involves reconciling several financial and operational trade-offs. This chapter discusses the challenges encountered when striving for the cost-effective manufacture of mAbs, the key process economic drivers, and the impact of scale and titer on downstream processing (DSP) economic trade-offs.

## 12.2 CHALLENGES WHEN STRIVING FOR THE COST-EFFECTIVE MANUFACTURE OF mAbs

The development of mAb therapeutics is a lengthy, costly, and risky business that is subject to stringent regulatory requirements. Decisions about the design of mAb processes and where to target process development efforts are complicated by the fact that they have to be made in an environment of uncertainty and constraints, as highlighted in Fig. 12.2.

### 12.2.1 Constraints

Biotechnology companies typically have a pipeline of drug candidates to manufacture for clinical trials and the market, but also finite resources, especially with regard to manufacturing capacity and budget, as well as regulatory constraints. The availability of sufficient manufacturing capacity is key, since pre-clinical studies, clinical trials, and product launch are all underpinned by the



**FIGURE 12.2** Key decisions, constraints, and uncertainties in biopharmaceutical drug development.

necessity to produce sufficient quantities of the prospective drug for evaluation or for the market. Typical demands for mAbs are milligram to gram quantities for trials and tens to hundreds of kilograms for commercial production. It is anticipated that a small number of antibodies will be required by the ton (e.g., Rituxan<sup>™</sup> and Avastin<sup>™</sup>) (10, 11). The potential repercussions of capacity constraints came into sharp focus when demand for the antibody fusion protein Enbrel<sup>™</sup> exceeded available capacity in 2000. This experience highlighted the huge cost and delay that occurs when capacity building hinders a company's ability to respond quickly to unexpected increases in market demand (9, 12).

Regulatory constraints have usually discouraged manufacturers from making process changes since product equivalence must be demonstrated and this can delay launch. Any significant deviation from the production protocol used to generate the trial material could invalidate all the clinical trial results with respect to the proposed commercialized product (13, 14). Extensive early development work could help to avoid the need for process changes. However, considering the risk of failure, most companies strive to minimize development times, which can result in suboptimal cell lines and processes. This is becoming increasingly unacceptable as reduced pricing flexibility, higher costs, and shorter product life cycles put pressure on companies to be more cost-effective.

### 12.2.2 Uncertainties

Clinical, technical, and commercial uncertainties provide further obstacles to the cost-effective management of mAb manufacture. Clinical uncertainties relate to the likelihood of success in clinical trials, with current success rates from Phase I to launch of only 18%–29% (1). These risks suggest that

companies should push four to five drugs into development each year to ensure one to two market successes per year. However, this poses tough operational and financial challenges. Dose requirements can also vary by an order of magnitude through the course of clinical trials. This significantly affects the number of batches required to meet clinical demands. These clinical uncertainties, combined with the large capital investment required, encourage companies to commit to creating manufacturing capacity as late and as cheaply as possible, without ever limiting their ability to produce in-demand drugs (9). Contract manufacturing and partnering have thus become common tools for the industry to meet capacity needs (9). Examples of technical uncertainties include the product titer during fermentation, the purification yield, and the duration of the manufacturing tasks. The impact of these factors on manufacturing costs is discussed in more detail in Section 12.5. Commercial uncertainties relate to sales forecasts and competition, where timely market penetration can mean the difference between a blockbuster drug and one that barely makes a profitable return on R & D expenditure (15).

As a result of these uncertainties, manufacturing schedules often have to be drawn up with insufficient knowledge of the likely dose range, the productivity of the cell line, and the market demand. For companies reserving space with contract manufacturers, they risk underestimating the time required, which could mean they have insufficient material for clinical trials, thus resulting in delays. On the other hand, companies may find that they do not have a drug candidate ready for manufacture at the required time, resulting in financial resources wasted on unused manufacturing capacity.

Given all these factors, the survival of a company can depend on the key decisions it makes during drug development (16). Hence, there is a growing emphasis on estimating and improving the cost-effectiveness of manufacturing strategies early in development so as to avoid significant business losses due to wrong decisions. The next section discusses methods for estimating key cost metrics.

## **12.3 COST DEFINITIONS AND BENCHMARK VALUES**

### **12.3.1 Capital Investment**

Given the relatively low potency of mAbs and the fact that they are capable of treating chronic conditions with large markets, the industry is predicting that inevitably, some antibody products will need facilities that can produce several hundred kilogram to ton quantities of antibodies to capitalize on this thriving market (10, 12, 17). Even so, biopharmaceutical manufacturing capacity is expensive, with construction times of 4–5 years to build, validate, and license a facility (9, 12, 18).

Recently, there has been significant activity in establishing antibody manufacturing facilities. These multiproduct facilities now reach sizes of

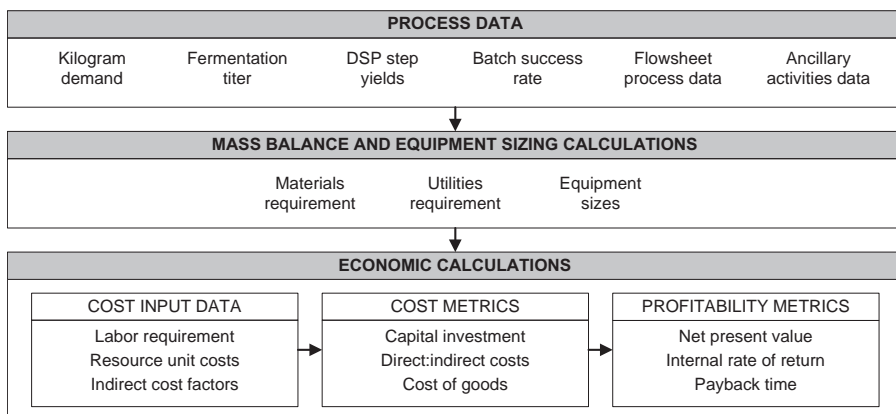
approximately 45,000 m<sup>2</sup> and have total bioreactor capacities of up to 200,000 L, typically achieved with multiple bioreactors of up to 25,000 L each. Based on these recent projects, current benchmark investment costs relative to facility size are \$7,130–\$17,000 per square meter and \$1,765–\$4,220 per liter for capacities in the range of 20,000–200,000 L (19). The wide ranges in the benchmark costs can be attributed to several variations among these recent projects relating to facility location, different ratios of upstream to downstream trains, and differences in the categories included in the published costs. In some cases, the costs and floor areas account for warehouses, support facilities, and office areas as well as the bulk manufacturing facility, and sometimes also include indirect costs. Despite these limitations, such benchmark costs are useful for deriving order-of-magnitude estimates at the early stages of design.

Although the benchmark data suggest that average costs for facilities with capacities of 20,000 and 200,000 L would be \$60 million and \$600 million, respectively, one would also expect economies of scale to influence the costs. The capital investment required for a large-scale facility can be approximated according to the cost of the pilot facility used to make the same product using the sixth-tenths rule, if scale-up is achieved through the use of larger equipment rather than increasing the number of process trains. Of course, if the capacity limit of, e.g., a chromatography column is reached, the accuracy of this estimation method will fall. Further limitations stem from the fact that not all individual equipment item costs scale according to the sixth-tenths rule; e.g., Remer and Idrovo (20) warned against blind use of the exponent value 0.6 and presented exponential scaling factors for 58 different types and sizes of bioprocess equipment where the exponent value ranged from 0.36 for computer-controlled fermenters to 1.00 for ultrafiltration rigs. On the other hand, if a facility is scaled up simply by increasing the number of process trains, then capital cost estimation becomes linear and can be approximated by multiplying the investment required for the pilot facility by the number of process trains.

### 12.3.2 Cost of Goods (COG) per Gram

Manufacturing COG values usually represent 15%–25% of sales (21–24). COG per gram values are highly dependent on both the scale of production and the titer. The lower the production rate (kg/year) and titer, and the smaller the scale, the higher the cost per gram. As such, unless these factors are known, it is difficult to compare values reported in the literature. Significant pressures exist to increase production scales for antibodies that are used at high doses (>1 g/patient/year) and have large potential markets (>500,000 patients) (25). This has triggered a drive to reduce commercial manufacturing costs by an order of magnitude from thousands to hundreds of dollars per gram (26) or even tens of dollars per gram (27).

COG is a useful measure for capturing changes in both the running and investment costs since it combines both the direct and indirect costs of



**FIGURE 12.3** Schematic showing the interaction between the key components of a process economic model.

alternative process flow sheets (Fig. 12.3). Direct (variable) production costs include raw materials and utilities, and indirect (fixed) costs such as depreciation/capital charges and maintenance are typically proportional to the capital investment. The distinction between variable and fixed costs is not consistent in the literature. For example, labor can be considered either directly related to operating activities or to be a fixed annual charge. In addition, for cost analyses in the literature, it is not always clear what is included under different cost categories.

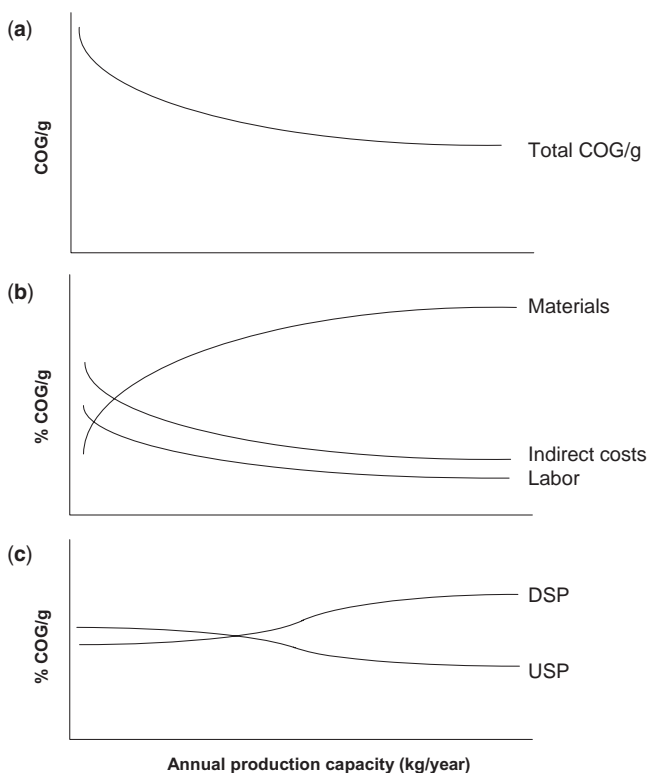
Current economic evaluations may not account for hidden costs in biopharmaceutical manufacturing. It is important to consider the costs not only of the key process steps but also of the ancillary activities such as media/buffer preparation, equipment cleaning, and quality/regulatory activities [e.g., documentation, quality control and quality assurance (QC/QA)] (8, 28–30) (Fig. 12.4). Furthermore, the time and cost of validation and regulatory issues are usually underestimated. For example, development costs relating to cleaning validation studies to demonstrate resin reuse stability may not always be captured in cost analyses. Another area of ongoing cost is environmental monitoring to maintain controlled environments. For example, heating, ventilation, and air conditioning (HVAC) systems are critical for controlling air particulate levels and air pressure differentials in different rooms so as to prevent contamination and to contain microorganisms used for production securely. It has been suggested that an extra cost category termed “general utilities” could be added to account for this (8, 31), calculated as a function of the floor area. Pugh (32) highlighted further hidden costs including process development effort, batch failure, risk, changeover time, learning curve effects, and plant utilization vs. product demand.



## 12.4 ECONOMIES OF SCALE

The relative importance of key process parameters on the overall economic feasibility of a process varies with both the scale of operation and the assumed titer. As annual output and scale increase at a given titer or combined with titer increases, the relative importance of different cost categories is expected to change as shown in Fig. 12.5. The overall COG per gram decreases, while in terms of the ratio of direct and indirect costs, the material costs rise considerably and dominate the COG per gram, whereas labor and capital-dependent costs (overheads) fall and hence represent a less-significant proportion of COG per gram. Similarly in terms of the ratio of upstream processing (USP) and DSP costs, the DSP costs become a major component of the COG per gram.

If the increase in annual output is also accompanied by titer increases, then the trends are expected to become even more pronounced. At small scales, fixed costs tend to dominate, and thus any changes in raw material costs will



**FIGURE 12.5** Economies of scale. Typical cost trends as scale increases for (a) total COG per gram; (b) material, labor, and indirect (capital-related) costs; and (c) USP and DSP operating costs.



have a minimal impact. However, as scale increases, the role of raw materials becomes more critical. It is therefore important to look at the distribution of the costs (upstream to downstream as well direct to indirect costs) at the scale and titer of interest in order to prioritize optimization efforts.

12.5 OVERALL PROCESS ECONOMIC DRIVERS

Efforts to lower COG per gram must be targeted at decreasing the overall batch costs (e.g., reducing raw material costs) and/or increasing the overall productivity (e.g., increasing process yields). Critical determinants of overall economic success include titer, overall yield from DSP, batch duration, batch success rate, and logistics. These key process economic drivers are discussed in more detail in the following sections. More specific DSP drivers, which become of prime importance when handling multigram per liter titers, are discussed in Section 12.6. These relate to resin/filter reuse and cycle limits, buffer and water-for-injection (WFI) demands, capacity, and flow rates.

12.5.1 Titer

The titer is determined by the chosen expression system and the degree of process optimization, which influences both culture longevity and productivity by adapting the media and feed composition, feeding regime, and environmental conditions. It significantly affects the number of batches required to meet demand in existing facilities or the production capacity to be incorporated into new facilities. Werner (33) provides a useful illustration of the impact of titer on the number of 10,000-L bioreactors required and hence the COG per gram to produce 250 kg/year (Table 12.1). Increasing the titer 10-fold resulted in the number of bioreactors falling by 15-fold and the COG per gram being reduced by an order of magnitude.

Most antibodies are currently produced using fed-batch Chinese hamster ovary (CHO) or mouse myeloma (e.g., GS NS0) cultures in stirred tanks (28, 34, 35). Antibody titers in such systems have improved dramatically over the past two decades—maximum industry titers in clinical manufacturing have

TABLE 12.1 Cost of Goods (COG) per Gram for Antibodies Produced at Different Titers in Mammalian Systems

Annual Production, kg/year	Titer, g/L	Production Bioreactor Capacity, L	COG/g, \$/g	COG/year, \$M/y
250	1	20,000	260	65
250	0.1	310,000	1500	375

Source: Data from reference 33.

increased from 0.02–0.05 g/L in 1985 to 0.5–2.0 g/L in 1995 to 3.0–5.0 g/L in 2005 (36–41). Further increases up to 10–15 g/L are anticipated over the next 10 years (40, 41). Increasing titers can free up existing capacity to allow for more products to flow through a facility on a multiproduct campaign basis or for smaller upstream facilities to be built in the future. The potential impact on DSP is discussed further in Section 12.6.

Although mammalian cells have emerged as the standard platform of mAb production, research into alternative systems such as the use of transgenic plants and animals has attracted attention in process development circles for applications requiring very large amounts of product. This reflects claims of competitive COG values, lower capital investment, the flexibility to modulate capacity, and the ability to assemble more complex antibodies, when compared to mammalian cell culture (41–45). However, it has been suggested that improved titers in mammalian cell cultures are dampening the prospects for such alternatives (46).

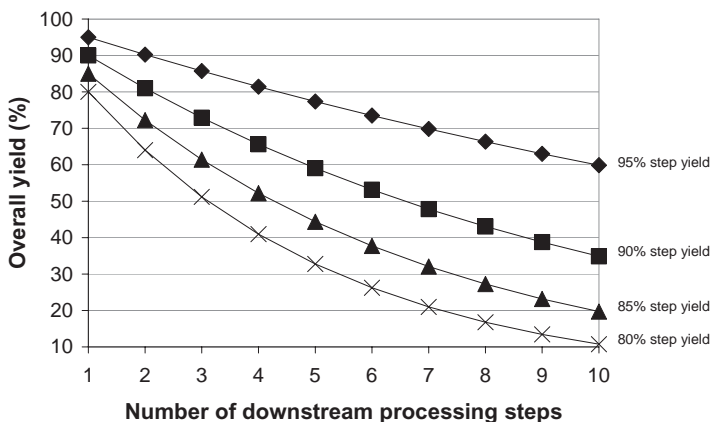
Companies are also using microbial expression systems, such as *Escherichia coli* and *Pichia pastoris*, for the production of antibody fragments, since glycosylation is not an issue. Such systems have historically achieved higher titers than mammalian cells, requiring shorter cultivation times as well as simpler media. Hence, one might expect production costs for antibody fragments to be lower than those of mammalian systems. Furthermore, recent efforts in glycoengineering *Pichia pastoris* to humanize the glycosylation of antibodies may allow more cost-effective production of whole antibodies in the future (47).

### 12.5.2 Overall DSP Yield

The overall DSP yield is a function of the individual step yields and the number of DSP steps as has often been demonstrated using the plot in Fig. 12.6. Improvements in step yields as well as reductions in the number of steps mean that typical overall process yields have increased from 40% to 75% in recent years (33, 48), with savings in COG values and investment as well as higher facility throughputs.

The impact of increasing step yields has been illustrated by Sommerfeld and Strube (35), where increasing the average step yield in a seven-step downstream process from 85% to 95%, which increases the overall yield from ~30% to 70%, results in a 40% reduction in the downstream COG per gram. Increasing step yields actually increases the equipment size or the number of cycles required, and hence the cost of the DSP steps since each step needs to handle a larger load (chromatography) or volume (filtration). However, since more product is produced per batch, the COG per gram typically falls with increasing yields.

Downstream processes for marketed antibodies typically consist of recovery by centrifugation and depth filtration or microfiltration followed by three chromatography steps with intermediate steps for viral clearance and buffer



**FIGURE 12.6** Overall yield as a function of individual step yields and the number of steps.

exchange (28, 34, 35, 48). To maximize productivity and minimize investment and running costs, it is advisable to keep the number of steps to a minimum (7, 33, 49). For antibody processes, this has encouraged the elimination of buffer exchange steps (diafiltration) that add little purification value by designing each chromatography step so that it can take the material eluted from the previous step where possible (33, 50, 51). The other option of reducing the number of chromatography steps is limited since traditionally, three or four steps have been required to meet regulatory requirements regarding satisfactory removal of adventitious agents (e.g., viruses) and process-related impurities (e.g., column leachables, host cell proteins, DNA). However, some companies have recently adopted processes that use only two chromatography steps while maintaining the desired purity levels (10). Obviously, this requires the second step, typically anion exchange, to have additional selectivity to replace the intermediate purification and polishing steps. In cases where the contaminant profile, pH, and conductivity provide the opportunity to adopt this simpler process, time and cost savings can be achieved.

Combining product recovery and initial capture into a single step has also been pursued to reduce losses and COG per gram while saving time. Expanded bed adsorption (EBA) chromatography offers a potential way of achieving this. The economic superiority of EBA over conventional recovery and packed-bed chromatography is largely dependent on the resin lifetime, dynamic binding capacity, and cost (52–54), as well as the likelihood of equipment failure where EBA can be prone to fouling with certain feed stocks. However, despite its potential, most manufacturers have not yet adopted EBA for mAb production (17), and this is due in part to the low resin capacities and lifetimes, as a result of more complex feeds and harsher cleaning-in-place (CIP) regimes relative to packed-bed chromatography (17, 55).

12.5.3 Batch Duration

As well as minimizing the number of process steps, other avenues exist for decreasing the batch duration and hence increasing a facility’s output. The most appropriate time to harvest a cell culture is often debated—harvesting earlier, e.g., at 10 days instead of 14 days, involves a trade-off between allowing more batches to be pushed through a facility per year and the lower output per batch. Furthermore, with higher titers on the horizon, there may be potential to move away from extended culture times of 2–3 weeks and to harvest earlier, as long as product quality is not compromised. Shorter fermentation times can also help to increase the utilization of DSP equipment/capacity.

12.5.4 Batch Success Rate

Batch failure can be due to contamination, equipment failure (e.g., filter integrity, resin fouling), or operator error. Contamination is a critical concern in biotechnology processes, e.g., fed-batch cultures have success rates in the range 95%–99% (30), while spin-filter perfusion cultures tend to have lower rates due to the extended culture periods increasing the likelihood of contamination and filter fouling. The impact of failure in a perfusion process will also depend on the QC/QA policy. Product lots that are harvested earlier than the contamination can often be processed if they have been checked by QC/QA, thus minimizing the cost of failure. Here the trade-off between the lower productivity and greater up-front investment required for fed-batch processes vs. the potentially greater operational risks of more complex perfusion processes needs to be quantified in terms of process economics (Table 12.2). Interestingly, fed-batch and perfusion processes may have similar COG per gram values if one assumes an equal chance of failure (30). However, if the probabilities of failure in perfusion processes are expected to be higher, this can lead to lower profits and in such cases, only changes in operational philosophy or equipment design can help to improve process robustness and hence economic feasibility. It is recognized that the choice between fed-batch and perfusion

**TABLE 12.2 Examples of Upstream and Downstream Process Economic Trade-Offs**

	Perfusion vs. Fed-Batch Processes	Membrane Chromatography vs. Packed-Bed Chromatography
Advantages	Investment ↓ DSP consumables cost ↓ QC/QA cost ↑	Investment ↓ Buffer cost ↓ Labor cost ↓ Cleaning validation cost ↓ Development cost ↓
Disadvantages	Risk of equipment failure ↑ USP consumables costs ↑	Separation medium cost ↑

*Note:* Advantages and disadvantages are given for the first option relative to the second option.

processes often depends on the experience within the company (56). A further point is that batch success rates are also subject to learning curve effects. As a result, switching to alternative unit operations can initially result in lower batch success rates and delays, which deter companies from making changes.

### 12.5.5 Logistics

A facility dedicated to the production of a single product will tend to give the lowest cost per unit of production (7). On the other hand, multiproduct facilities such as those used to produce clinical trial material require increased flexibility, more equipment in use only part of the time, the introduction of turnaround times, as well as dedicated resins and filters, leading to higher costs per unit of production.

Pooling harvests also alter the process logistics, and it is important to determine the pooling interval that minimizes costs and uses resources more effectively. Pooling can potentially reduce labor and quality costs since fewer staff are required for recovery and fewer bulks require extensive quality testing (49). However, these savings need to be weighed against the increased costs of larger DSP equipment or additional cycles to process the larger volumes. Product stability also influences the choice of pooling strategy. A less-stable product would require more rapid processing and hence frequent pooling of the broth. For example, no significant differences in expected costs were found for different pooling strategies used with 1000-L perfusion culture processes (29). However, when the impact of batch-to-batch variability in titers and DSP yields was taken into account, shorter pooling intervals were shown to be more favorable with a reduced risk of exceeding the budget and less chance of failing to meet the desired annual output. The benefits of pooling harvests will also depend on the scale of operation since the importance of direct and indirect costs varies with scale.

## 12.6 DSP DRIVERS AT HIGH TITERS

As titers increase to 10–15 g/L, the COG per gram is expected to decrease as long as the purification costs do not negate the cell culture gains. With increasing titers, the ratio of upstream to downstream costs shifts so that the downstream costs become more dominant. For example, Sommerfeld and Strube (35) calculated that increasing the fermentation titer 10-fold from 0.1 to 1.0 g/L caused the ratio of upstream to downstream costs in their process to drop from 55:45 to 30:70. This shift reflects the fact that USP costs are inversely proportional to titer, but the same is not true for DSP costs (35). Increasing the titer so as to satisfy larger market demands increases the protein load on chromatography steps, resulting in an increase in the number of cycles or additional investment in larger columns; this also produces larger volume loads on any subsequent filtration steps leading to longer filtration times or a need for larger

areas. All these factors increase the downstream operating costs per batch. However, the overall COG per gram can still fall if the increase in overall productivity outweighs the increase in downstream costs. Accordingly, as titers increase further, the DSP steps will become major contributors to the overall COG per gram and hence offer the greater potential for improvements and cost savings. Consequently, the downstream yield and material costs become significant cost drivers. DSP bottlenecks can encourage increased investment in larger equipment and longer batch durations. This in turn results in increased running costs and decreased productivity, and hence suboptimal COG per gram values. The implications of DSP drivers and bottlenecks are discussed below.

### 12.6.1 Material Reuse and Lifetime

In DSP, the distribution of raw material costs depends not only on the scale but also on whether or not resins and filters are reused. When treating these material components as single-use/disposables, resins and filters tend to dominate the material costs. Similar patterns can be seen in clinical manufacturing since these materials remain product specific. The impact of this operational strategy on the overall COG per gram can depend on both the scale and the phase of development. The use of downstream consumables such as disposable resins and membranes in a 200-L antibody process supplying early-phase clinical trials can provide both financial and operational savings (8, 28, 57). Here, the trade-off between the advantages of disposables, such as lower capital investment, faster turnaround between campaigns, reduced cleaning validation, and increased flexibility, was compared against the potential disadvantages, such as increases in material demands, validation of leachables, and reliance on suppliers. It has been reported that DSP using disposables can become a major disadvantage at the 10,000-L scale (58). This can be attributed to the fact that economies of scale result in a disproportionate effect on raw materials (52). Consequently, raw material savings become more important for any process as scale increases.

The reuse of resins and filters involves a trade-off between reduced material costs and increased cleaning validation costs to determine the number of reuses with consistent performance. The higher the component cost, demand, or number of process steps, or the lower the validation costs, the greater the incentives to adopt filter/resin reuse (59). Protein A chromatography resins are often quoted as dominating the raw material costs of DSP steps because they are over an order of magnitude more expensive than ion-exchange resins. Bioreactors at the 10,000-L scale operating at a titer of 1 g/L require Protein A resin costing in the order of \$4–5 million (60). Consequently, Protein A resins tend to be used in smaller quantities with multiple cycles despite the complications of reuse validation (55, 60). The reuse of Protein A resins can dramatically reduce their relative contribution to costs, making the costs

associated with filters much more prevalent. In particular, virus filtration can become a more dominant component of purification costs (61).

For most companies, Protein A remains the workhorse of purification processes due to its high selectivity, achieving 95%–97% yields and >99% purity (2, 28, 34, 41, 61, 62) (see Chapter 4). While replacing Protein A with a cheaper resin can reduce raw material costs, few resins can compete with its selectivity at present (63, 64), and its replacement could therefore reduce productivity, offsetting any cost benefit. One expensive but high-yielding step may be less costly than several less expensive but low-yielding steps (7). Having said that, some commercial antibodies have been produced without Protein A steps (28, 34, 35) (see Chapter 5).

### 12.6.2 Buffer/WFI Demands

As the reuse of filters and resins increases, buffers (chemicals and WFI) can account for a surprisingly large proportion of the costs, in some cases greater than the cost of resins and filters. For example, at a fermentation scale of 20,000 L, approximately 140,000 L of buffer is required (65). The difficulty in calculating buffer costs partly reflects the large differences in the estimated costs of WFI, with values of approximately \$0.2 per liter suggested for in-house generation and up to \$3 per liter for vendor or contract manufacturing organization (CMO) charges (66). Buffer costs have been quoted as varying between \$2 and \$12 per liter (67). Efforts to reduce the volume and number of buffers required can potentially lead to savings, as naturally occurs when moving from a three-step chromatography platform to a two-step one.

More recently, an additional raw material has come into the equation—bags. Disposable bags have been adopted by the industry as buffer and product-hold vessels instead of stainless steel tanks (8, 28, 57, 68, 69). This can offer overall COG benefits by reducing the investment in hardware and hence overheads (indirect costs), which tends to outweigh the increase in raw material costs. For example, a study of the impact of disposable bag technology for media and buffer preparation, as well as for media, buffer, and product hold in a 1000-L perfusion process, suggested savings of 41% and 17% in the capital investment costs and COG, respectively, compared to using stainless steel vessels (69).

As increasing titers demand larger downstream equipment and/or additional cycles, the requirement for buffers and WFI will also increase. In existing facilities, this can present retrofit challenges if demands exceed the capacity of the buffer preparation suites and WFI storage tanks or the rate of WFI generation. The use of buffer concentrates and in-line buffer mixing can help to reduce the tank size and hence floor space required for buffers (11). However, there is also increased demand on vessels to hold product streams between steps (10, 11, 70) which can exceed 10,000 L at high titers and large scales.



### **12.6.3 Chromatography Capacity**

The greater the mass load during chromatography, the greater the likelihood that the practical capacity of chromatography columns will be exceeded (12) where the current limit of column diameters is 2 m. Under these circumstances, multiple cycles may be required and there is a risk that the DSP time will exceed the bioreactor time. This will reduce the potential throughput of the facility and hence impact on the COG per gram. These large columns can also pose installation challenges in existing facilities if there is insufficient floor space and if they cannot fit through doors (71).

## **12.7 PROCESS ECONOMIC TRADE-OFFS FOR DSP BOTTLENECKS**

Current efforts to avoid DSP becoming a bottleneck when handling larger masses include intensifying existing processes by enhancing capacity and speed. Such approaches can mitigate the need for extra investment in equipment and can improve the process economics. Recent improvements in resins that allow higher binding capacities, higher flow rates, and longer cycle limits have facilitated process intensification efforts. A further approach that has been under investigation is the use of alternatives to column chromatography such as membrane chromatography, precipitation, and crystallization. However, with each of these approaches, there are trade-offs and uncertainties that need to be evaluated to assess the impact on overall process economics. These are summarized in the following section, and discussed in more detail in Chapters 14 and 15.

### **12.7.1 Chromatography Resin Dynamic Binding Capacity**

Increasing the resin binding capacity reduces column size requirements, resin volumes, and buffer consumption (for equilibration, washing, elution, regeneration, and CIP) per batch. Novel resins have capacities that are more than twice those of the first-generation resins (61, 72). This can lower consumables costs, which are a major component of COG per gram at higher titers and demands (35), although obviously the impact on the consumables costs will depend on the price differential between first- and second-generation resins. Given that affinity resins are more expensive than ion-exchange resins, increasing the binding capacity of affinity resins can have a greater influence on COG per gram. With the cheaper ion-exchange resins, Sommerfeld and Strube (35) highlight that a trade-off exists between the less-pronounced drop in consumables costs and the increase in labor costs, which becomes more important at higher binding capacities due to the longer processing times. However, given that most of the novel resins also allow higher flow rates, this may not be an issue.



### 12.7.2 Chromatography Flow Rates

The first generation of cell culture-based mAb processes used compressible chromatography resins. However, these impose severe limits on usable bed heights and flow rates when considering the expected increases in titer and scale (71, 73). A move away from compressible resins and toward rigid resins that can handle higher flow rates reduces process cycle times and turnaround times, and increases productivity (61, 71, 72). Flow rates with rigid resins can be three to five times faster than conventional compressible agarose resins (61, 72). However, their use can also lead to increased buffer demands, higher pressure drops, packing complications, and shear stress (71). Recently, new ion-exchange resins have been developed that can handle high flow rates (700 cm/h) and low back pressures (<3 bar). If the productivity increases outweigh the increased buffer demands, then this will positively influence the COG per gram.

### 12.7.3 Chromatography Resin Cycle Limits

As mentioned in Section 12.6.1, resin and filter reuse can have a significant impact on process economics at higher scales if the materials are expensive, despite the CIP and cleaning validation costs. Reuse also reduces the frequency of column packing, which is time-consuming and costly when carried out on a large scale (72). New resins are becoming available with increased stability when exposed to the harsh chemicals used for CIP; hence, they have longer cycle limits and can contribute to reducing the raw material costs.

### 12.7.4 Platform Processes

Platform processes provide a generic approach to antibody production that greatly reduces the development time while streamlining the regulatory aspects of processing. They represent a useful starting point for customization, depending on the antibody being manufactured. Advances in resin properties have also allowed platform processes to emerge with two rather than three chromatography steps (10, 11), which can help to alleviate DSP bottlenecks in existing facilities since a two-step process occupies less floor space and consumes less buffer (Section 12.6.2). Through such process intensification methods, Kelley (10) predicted that a platform consisting of two chromatography steps with high-capacity resins would be able to handle an annual output of 10 tons. Newer resins with the combined attributes of longer lifetimes, higher flow rates, and improved dynamic binding capacities will obviously lead to improved platform processes for antibodies and will contribute to significant reductions in DSP costs (11, 72).

### 12.7.5 Alternatives to Chromatography

Research into alternatives for column chromatography focuses on methods that can efficiently handle increased amounts of both the product and impurities (e.g., host cell proteins and antibody aggregates/isomers). Ideally, these alternatives should achieve a separation power equal to that of column chromatography while reducing the COG per gram (74). When assessing the cost-effectiveness of these alternatives, it is important to consider not only the equipment size and resource consumption but also the development and validation costs required.

Processes for the purification of mAbs typically employ cation-exchange chromatography to bind the product and anion-exchange chromatography to bind impurities such as DNA and host cell proteins. Membrane chromatography operating in flow-through mode is emerging as a popular alternative to anion-exchange chromatography in mAb purification because of the rapid operation, ease of scale-up, and cost savings as listed in Table 12.2 (see Chapter 14) (48, 66, 75–77). However, the low binding capacity of membranes has thus far limited their economic potential in bind-and-elute operations such as cation-exchange chromatography. For anion-exchange applications, the dominant component in the distribution of raw material costs shifts from buffer consumption in packed-bed chromatography to membrane use, as a membrane suitable for processing a batch of several thousand liters can cost thousands of dollars and is disposable and not reusable. The key economic trade-offs for anion-exchange applications therefore depend on whether the savings in buffer, labor, and overheads outweigh the high cost of the membranes. Critical variables that will affect the outcome of this cost comparison are the relative differences in the handling capacities assumed between anion-exchange membranes and resins, which dictate the sizes required, and the assumed WFI/buffer costs. Higher values for these variables increase the economic attractiveness of membrane chromatography (66). The pace at which resin and membrane capacities improve will contribute to which operation secures its place in future platform processes. In cases where packed-bed and membrane chromatography offer similar COG per gram, the real cost advantages may be in the development and validation costs, which are significantly reduced with membrane chromatography since there is no column packing or cleaning validation (66).

More traditional methods such as precipitation and crystallization are also being explored for high-capacity scenarios. However, more technical studies are needed to assess their suitability for mAb platform processes before meaningful economic studies can be carried out (see Chapters 13 and 20).

## 12.8 SUMMARY AND OUTLOOK

As demand and titers continue to increase for mAbs, the DSP costs will come to dominate the COG per gram with the DSP handling capacity representing

a potential bottleneck that could reduce productivity. As illustrated in this chapter, these factors have encouraged a shift in development efforts toward new DSP solutions that improve the process economics and alleviate bottlenecks. Consequently, the industry is taking advantage of improvements that affect the critical process economic drivers by looking to improve the overall DSP yield and to reduce the batch duration using platform processes based on two chromatography operations without intermediate buffer exchange steps. It is also seeking to increase DSP capacity by taking advantage of improvements in chromatography resins that allow increased throughput over shorter times, and to lower buffer demands and validation costs using new technologies such as membrane chromatography. All of these improvements will be important for both facilities that already have large bioreactor capacities installed and for newer facilities that will probably be built with smaller capacities given the higher titers, and with flexibility in mind to allow rapid turnaround between campaigns for multiple products. Furthermore, if cheaper and faster expression technologies, such as glycoengineered *Pichia pastoris*, become more widespread, there will be an even greater spotlight on DSP costs. Although new DSP approaches may present complex and challenging problems, it is anticipated that this line of enquiry will dominate studies in the near future so that more cost-effective mAb platform technologies can evolve. However, with each of these new approaches, there are trade-offs and potential risks that need to be evaluated to assess the impact on process economics. The capacity to cost such alternatives provides a common basis for such decision making and as such will prove a vital tool for bioprocess designs in the future. A final point is that process economics can also be dramatically improved if the potency of mAbs is increased; recent efforts in this area are an encouraging sign for the future.

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# 13

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## DESIGN AND OPTIMIZATION OF MANUFACTURING

ANDREW SINCLAIR

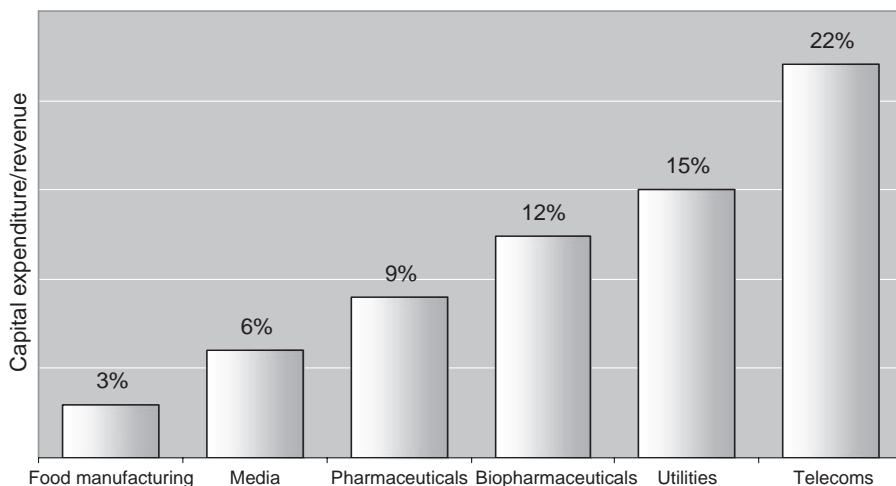
### 13.1 INTRODUCTION

In the last 10 years, there has been substantial investment in the manufacturing capacity of products based on monoclonal antibodies (mAbs). That capacity has increased from about 200,000 to 2,000,000 L, reflecting how the industry has matured. The capital investments are large and the costs of the drugs are high relative to existing treatments. In addition, healthcare providers are putting pressure on pharmaceutical companies to reduce costs. As a consequence, the manufacturing environment for mAbs is changing. It is important to understand how these pressures arise and how they will impact the next generation of mAb-based products in the context of development and manufacturing.

To better understand the capital structure of the industry, it is useful to compare the level of capital intensity in the biopharmaceutical industry relative to other sectors. Capital intensity, calculated as a percentage, is capital expenditure divided by total revenue for a given period. Morgan Stanley has published comparative data for the pharmaceutical sector (1) and the US Department of Commerce carried out an assessment for the biotechnology sector in 2001 (2).

By comparing the two sets of data (Fig. 13.1), it is clear that the biopharmaceutical sector has a much greater capital intensity at 12.4% compared with general pharmaceuticals at 9%. This does not bode well for an industry where there is pressure to reduce prices. If process manufacturing capital





**FIGURE 13.1** Capital intensity for different industries.

requirements do not change, then the capital intensity value will increase further as healthcare providers, competitive products, and generics start to put increasing pressure on biopharmaceutical pricing.

The high capital intensity partially explains high drug manufacturing costs, but it also presents the industry with a challenge that must be taken seriously—reducing the cost of manufacture. Another factor in drug pricing is the cost of development, and the time taken to develop drugs [currently about 7 years (3)]. There is much discussion on reducing development times, but much of this is focused on the clinical trials (the critical path in the product life cycle) where the industry will look to accelerate drug development through expanded use of information technologies in clinical trial management (4). However, the trends for the cost and duration of development are both increasing.

Given this background, the emphasis is on developing cost-effective, robust, and compliant processes where process development will not feature on the critical path. This is made more difficult by the increasing costs and duration of product development. There are three ways to approach this challenge.

1. Develop better processes.
2. Apply novel/new manufacturing technologies.
3. Operate facilities more effectively.

This chapter will consider those modeling techniques that can be used to support the achievement of cost-effective processes. With the appropriate use of modeling tools, we can gain insight and knowledge on the best ways to design manufacturing processes.

### 13.2 PROCESS DESIGN AND OPTIMIZATION

High capital costs, complex processes, and long product development cycles result in high manufacturing costs in the biopharmaceutical industry. There is pressure to reduce these costs, and this can be achieved by considering the whole product life cycle and by understanding what modeling tools can be used to help gain insight and to guide decision making. The key benefits of the modeling techniques are to develop processes that are

- simple and robust;
- able to employ the most effective technology;
- able to operate effectively in an actual manufacturing facility;
- robust and compliant.

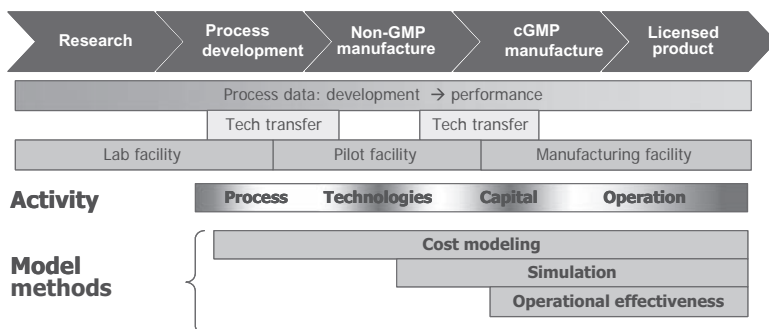
In addition, we are looking for tools that can manage change within existing facilities so that they can be used to support process improvements, operational improvements, and the introduction of new products.

A product starts off in research and, if successful, is manufactured for use in the clinic. During the life cycle of the product, opportunities to reduce the cost of manufacture become scarcer as the product matures. By breaking the product life cycle down into discrete stages (Table 13.1), cost objectives and the modeling tools/techniques that support the objectives can be identified.

Process development can be defined as the start of the product life cycle, and it is at this stage that the manufacturing process is defined in terms of unit operations (discrete manufacturing operations). There are significant

**TABLE 13.1 Modeling Requirements**

Stage	Objectives	Tools
Process development, early phase	Rapid development Minimal development effort	mAb “platform” process Cost models
Process development, late phase (for manufacturing)	Cost-effective robust process Optimum process Optimal process conditions Best manufacturing technology Good manufacturing fit Manufacturing strategy	Cost models Process unit operation models Process/facility simulations
Facility design (for a new or existing operation)	Minimize impact on facility Minimize capital	Process/facility simulations
Facility operation	Best operational performance Manage change	Cost models Process/facility simulation Operational effectiveness



**FIGURE 13.2** Product life cycle modeling approaches. USP: Upstream processing; DSP: down stream processing.

pressures on process development to be rapid (not on the critical path of a clinical program) and to reduce the cost of development. The cost pressures are related to clinical risk insofar that a product entering clinical trials (5) has a high probability of not reaching approved status (there is a high attrition rate). Consequently, there is no incentive to put a lot of effort into developing an efficient process early on. mAbs as a class of products are similar in nature and lend themselves to fitting into a generic approach to purification (see Chapter 12) with a second-generation process being developed once product success is assured. It is the goal of modeling, together with automated and rapid process development techniques, to remove the necessity for first- and second-generation processes by developing the optimum process first time round, within the constraints of the clinical program.

With respect to the product life cycle, the clear message is that as a product moves from research into manufacturing, the scope for improving the cost effectiveness of the manufactured product diminishes. The later stages of the life cycle are more about efficient operation of facilities.

To illustrate this point, Fig. 13.2 shows the relationships between process information and facilities superimposed on the typical product life cycle. Process information is the glue linking all the modeling tools, and the facility required for manufacture is the process data set generated in development and utilized in manufacturing.

The key to maximizing the benefits of the process sets is knowledge management within and between products (6). Currently, there is no consistency in approaches to managing and using process information. It is recommended that industry considers moving to a consistent, standard terminology for representing bioprocesses. This is essential in order to create a data model that can be applied broadly. Such a general model for batch manufacturing already exists and is defined by the Instrumentation, Systems and Automation Society—the ISA-88 Standard for Batch Control point. It is widely used for control systems and automation (7, 8). Two aspects of ISA-88 are especially useful for defining a bioprocess model: a separation of process requirements from equipment capability and a modular design approach. The concept of

defining a process in dimensionless terms is inherent to scale-up and facility-fit assessments, and the practice of defining bioprocesses as a sequence of unit operations is essentially a modular design.

Ultimately, the goal of the knowledge management model is to enable feedback from manufacturing performance to guide process development and to create a model that supports continuous improvement and better process understanding. Various modeling tools are commonly used in the industry to gain a better view of the effect of process development decisions on manufacturability, and the data model should support more effective use of these modeling tools. By enabling assumptions and relationships defined in the model to be compared to historical process data, modeling tools can be refined and improved continually. For example, cost models can assess the impact of process development decisions on manufacturing costs, and simulation models can assess facility fit and overall manufacturing performance and resource utilization.

### 13.3 MODELING APPROACHES

#### 13.3.1 Cost Modeling for mAb Manufacture

Cost models in the broadest sense can be applied widely as tools to analyze processes and manufacturing options and to support decision making. They have been used in our industry to

- assess the cost of outsourcing;
- evaluate and screen process development options;
- help develop capacity and expansion strategies;
- compare existing and novel processing/manufacturing technologies.

In this section, the different approaches to cost modeling are considered, and the merits of each approach are described and compared.

**13.3.1.1 Basic Accounting Principles.** Most cost models draw on the principles of financial and management accounting to assess the cost impact of different investment and operating decisions. In traditional financial accounting, the impact of manufacturing changes is seen primarily in three of the four basic financial statements: the balance sheet, the income statement, and the cash flow statement. The balance sheet shows the balance between the company's assets and its liabilities and equity at a given point in time. Manufacturing-related activities such as equipment purchases and increases in inventory levels will be apparent on the asset side of the balance sheet, as well as the corresponding decrease in cash or increase in debt/equity financing required to support these assets. In contrast to the "snapshot" view offered by the balance sheet, both the income statement and cash flow statement show

the flow of money in and out of the company over a period of time. The income statement, also referred to as a profit and loss (P&L) statement, shows the translation of revenue into net profit for the company after expenses are taken out for a given financial period. Because the income statement is based on accrual accounting methodologies, the revenue and expenses shown in the income statement do not correspond directly with the actual cash received and spent by the company during the same time period. This information is instead shown in the cash flow statement, which is often used to assess the short-term financial stability of a company. The cash flow statement is especially important for cost models as the timing of cash flows related to a specific project is commonly used for financial models such as net present value (NPV) and internal rate of return (IRR).

For manufacturing, the most significant line item on the income statement is the cost of producing goods for sale [referred to as cost of goods (CoG) sold or cost of sales], which is shown directly below the net sales revenue. Subtracting the CoG sold from the sales revenue results in a company's gross profit, which is an important measure of operating performance. The CoG sold is also related to inventory valuation, as the basic equation for calculating the book value of inventory is

$$\text{Beginning inventory} + \text{net purchases} - \text{CoG sold} = \text{ending inventory} \quad (13.1)$$

By separating the direct costs involved in producing goods for sale from other expenses, such as selling, general, and administrative (SG&A) expenses, research and development (R & D) expenses, interest, and taxes, it is possible to evaluate manufacturing performance as a distinct measure that contributes to overall business performance. This distinction is particularly important from an executive management perspective because improvements in manufacturing performance that result in increased gross margin are made visible, even though the bottom line of company net profit may remain unchanged or may even fall because of increased expenses elsewhere in the company. The components of the CoG calculation, which include labor, materials, and overheads, will be considered in detail in the next section. A robust, well-structured cost model enables managers to have a better insight into the key cost drivers of the manufacturing process as well as the sensitivity of overall CoG to changes in these key parameters. These models enable the cost impact of implementing different technologies to be evaluated, as well as the effect of process changes such as increasing product titers and yields, and these can be validated with financial accounting data. It is worth noting that some management accounting techniques such as life cycle cost analysis and activity-based costing are also incorporated in manufacturing cost models in recognition of the significant effect manufacturing efficiency has on the average CoG. In particular, the number of successful production runs per year and the cost of facility downtime and batch failure often have a much greater effect on overall manufacturing costs than changes in raw material or labor costs.

**13.3.1.2 Project Appraisal.** Some of the typical methods for evaluating projects are NPV, IRR, and return on investment (ROI). All three methods provide useful tools for decision making, particularly for capital investment and project approval. NPV and IRR are particularly popular for evaluating potential long-term projects because they account for the up-front investment required as well as the timing of future cash flows and the risk and/or opportunity cost of the project.

The basic calculations for NPV and IRR are

$$\text{NPV} = \text{initial investment} + \sum_{t=1}^N \frac{(C_t)}{(1+r)^t} \quad (13.2)$$

$$\text{Initial investment} = \sum_{t=1}^N \frac{(C_t)}{(1+\text{IRR})^t} \quad (13.3)$$

where initial investment is the cash invested at the beginning of the project ( $t = 0$ );  $t$  is the unit of time (measured in years);  $N$  is the lifetime of the project in years;  $C_t$  is the net cash flow for each year of the project, and  $r$  is the discount rate, also referred to as rate of return, interest rate, hurdle rate, or cost of capital.

For NPV calculations, one of the most crucial considerations is the discount rate. The rate at which future cash flows are discounted to represent their present value is generally based on a required rate of return, which for financial planning purposes is typically the rate that would be expected from an investment of comparable risk. Many companies choose to use their weighted average cost of capital (WACC) as the discount rate for all project appraisals, as this value reflects the company's financing structure and risk profile, whereas others choose to use higher discount rates for riskier projects. For biotechnology investments, the required rate of return is often quoted at 20% or higher to reflect the high risk and uncertainty associated with positive cash flows in this business sector.

When interpreting NPV, the accepted logic is that projects that have a positive NPV will add value to the company and should be undertaken, whereas projects with a negative NPV should be rejected. If a project's NPV is 0, then the company should be indifferent to the project as it will neither add to nor subtract from the value of the company. The decision to go ahead with the project should therefore be based on other criteria such as strategic benefits that are not captured in the NPV analysis.

IRR analysis offers an alternative view to the same series of project-related cash flows. In financial terms, IRR is the annualized compound rate of return from the initial investment, and in mathematical terms, the IRR is the discount rate that results in an NPV of 0 for a given series of cash flows. In general, if a project's IRR is greater than the company's average cost of capital, then the project will add value and should be accepted.

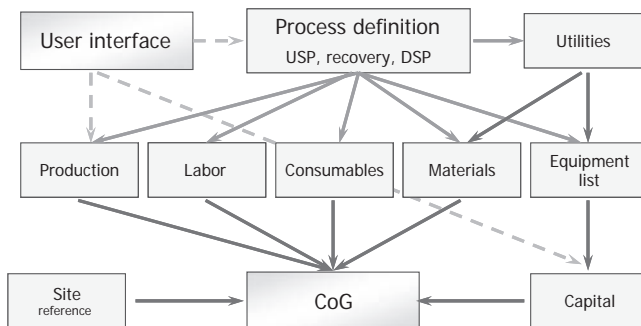
Although both NPV and IRR are based on the same financial principle, there is some debate about which measure is most appropriate for evaluating long-term projects. Academics tend to prefer NPV as it directly measures incremental value to the company whereas many executives prefer IRR as it indicates a percentage rate of return. Both measures are powerful tools, but should be used with caution. As investment decision tools, IRR is best used to evaluate a single project rather than to compare alternative projects, whereas the discount rate chosen should be considered carefully when using NPV to evaluate a single project.

Another method of evaluating expected return is ROI, which is useful for a variety of considerations such as short-term projects. In simple terms, ROI is the ratio of money gained or lost as a result of an investment to the money invested. For example, if a \$500 million capital investment resulted in an incremental revenue of \$150 million and incremental expenses of \$100 million in the first year, the ROI would be  $(\$150 \text{ million} - \$100 \text{ million}) / \$500 \text{ million} = 10\%$  ROI. ROI may be based on cash flows or net income/loss and may be based on a single point in time or may account for the present value of gains/losses.

Of the methodologies described below, CoG is by far the most commonly used method. Although not the most rigorous, it does have the merit that most people in the industry understand this approach. CoG should not be used where there is a need to understand the interplay between the expenditures and the project risk. NPV methodology is the best technique to analyze alternative technologies and manufacturing strategies, as it can account for the impact of delays in expenditures and can properly account for the time value of money. NPV is not commonly used, but it is rigorous and can be used to support decision making.

**13.3.1.3 CoG Modeling.** To illustrate this approach, we examine a spreadsheet-based and modular approach initiated at BioPharm Services that has been adopted for the implementation of over 50 CoG models. The cost model is configured as modules (i.e., capital, materials, consumables, labor, etc.) using the worksheets in a Microsoft Excel workbook. Figure 13.3 shows the relationship between the various worksheets and the cost components that constitute the overall CoG. The spreadsheet methodology has the advantage that it is scalable, flexible, user-friendly, and transparent.

The use of CoG is a fairer comparison than capital cost because it accounts for all differences in facility throughput, material costs, labor costs, etc. The indirect (fixed) cost consists of the capital charges, insurance, and taxes, while the direct (variable) costs include the consumables, materials, and labor. Figure 13.3 shows the component items that contribute each of the cost categories, e.g., the consumables class includes the column gel, filters, and single-use bags. A brief description of the key modules is provided in this section, including details of the user inputs that form the basis of the model and the calculation method.



**FIGURE 13.3** Structure of the CoG model. USP: Upstream processing; DSP: downstream processing.

**13.3.1.3.1 User Interface.** The user interface enables rapid assessment of the impact of predefined key model input parameters for what-if analyses. Examples include changing the production scale and switching between different manufacturing options. Such an interface enables changes in the underlying data to be reviewed rapidly and conveniently.

**13.3.1.3.2 Process Definition.** The operating costs are determined by the process definition (includes upstream cell culture, recovery, and downstream purification operations), which forms the core of the cost model. A detailed breakdown of each unit operation into subunit steps is captured. The key parameters include the type and total quantity of solutions/utilities used, the number of preparation/hold operations, the volume of vessel/bag used, the operating time, the elapsed time, the labor headcount for each substep, and the manual hours needed to carry out the operation.

**13.3.1.3.3 Production.** The user-defined parameters in this module consist of the campaign length, maintenance duration, and validation time. These parameters are used to compute the total available operating time. The limiting step per batch for cell culture, recovery, and purification is calculated. This figure is then used to calculate the process bottleneck and the annual number of production batches generated, given the particular operating constraints.

**13.3.1.3.4 Equipment List.** The objective of this module function is to generate a priced equipment list and a total purchase cost estimate for the major and supporting equipment items in the manufacturing option. If the cost of a piece of equipment is unknown, a cost estimate can be deduced from a known cost for that type of equipment and the ratio of the capacities raised to an index value (9).

**13.3.1.3.5 Capital Charge.** The charge on the total fixed capital investment is factored into the cost model as an amortized annuity charge, termed the



**TABLE 13.2 Capital Estimation**

Category	Description
A. Fixed equipment costs	Total equipment purchase costs
B. Capital estimates	
Installation	$\alpha\%$ of A
Pipework*	$\beta\%$ of A
HVAC*	$\chi\%$ of A
Instrumentation and control*	$\delta\%$ of A
Electrical power	$\epsilon\%$ of A
Process utilities	$\phi\%$ of A
Building*	$\gamma\%$ of A
Fit-out	$\eta\%$ of A
C. Total cost of works	A + B
D. Others	
Validation	$\lambda\%$ of C
Fee	$\mu\%$ of C
E. Total capital investment (TCI)	C + D
F. Annuity charge (capital charge)	PMT (cost of capital, period, E, future value <sup>a</sup> E)

\*Discount factors included for disposable.

capital charge. The annuity capital charge is the payment for a loan based on constant payments and a constant interest rate. In the model, the capital charge is calculated based on an 8-year period, a value of 12% for the cost of capital (the interest rate for the capital investment) and a future value (the residual value attained after the last payment is made, here expressed as a percentage of the capital investment) of 10%, which are typical values used in the industry. Table 13.2 shows the capital factors that constitute the total capital investment (TCI). The capital elements (e.g., installation, pipework, electrical power, building, etc.) are determined as using benchmarked percentages of the total equipment purchase costs. Discount factors have been incorporated into certain capital estimates to account for the use of disposables.

**13.3.1.3.6 Materials.** Process materials include media, buffer solutions, and cleaning chemicals (i.e., caustic and acid), which are made up from solid ingredients using purified water (PW) or water for injection (WFI). The process equipment for cleaning includes the preparation and hold vessels, bioreactors, housings, and skids. The molecular weight, pack size, pack unit, and cost per pack for the raw chemicals are user-defined variables, which are used to determine the unit cost per liter for the solutions. The costs are obtained from vendors or suppliers. The compositions of each solution are indicated by specifying the composition of each of the chemicals in the solution. The total volume per batch for each type of solution and cleaning chemical is reported. The amount used is multiplied by the unit cost to calculate the total cost per batch.

*13.3.1.3.7 Consumables.* The user-defined consumables costs include filters, chromatography resins, chromatography membrane devices, and the disposable preparation and hold bags. The total consumption of consumables per batch is determined. For disposable consumables, the costs per batch are calculated by multiplying the number of each type of consumable used and the unit cost. In the case of reusable consumables, the cycle limit and cycles per batch are used in the calculation.

*13.3.1.3.8 Labor.* Labor headcount is estimated for each unit operation within the cost model. The assignation of labor costs to a manufacturing batch is based on the allocated time that the direct operation staff spent in production on a particular batch. The number of manual hours required per batch is calculated for the following categories.

- Production—the direct production operators and supervisors required in the main process and supporting activities such as buffer preparation and cleaning. The labor hours for the direct production operators are used to estimate the manual hours required for supervisors by applying a benchmarked percentage.
- Quality—the staff required in validation, quality assurance (QA), and quality control (QC). These are estimated using a function of the direct production labor using figures from benchmarking studies.
- Others—logistics and general management. The labor requirement is calculated using a function of the direct production personnel.

The different categories of personnel, their annual salaries, and overheads are user input parameters. The wage per hour for each personnel category is calculated using the annual salary, operating weeks per year, operator hours per week, and overheads. The number of manual hours and the hourly wage are then used to determine the total labor costs per batch.

*13.3.1.3.9 Utilities.* The cost model collates all process uses of PW and WFI to provide minimum estimates of the capacities of the PW and WFI generators and the volume of the storage vessels. The input parameters required for the estimation include effective utilization, still blowdown, and fill time for storage tanks. The costs of the utility systems are added as fixed costs to the total equipment purchase costs.

*13.3.1.3.10 Site.* Other running costs for the manufacturing process include

- insurance and others—estimated as a function of the total fixed capital investment;
- engineering and spares—determined as a function of the direct production labor;
- utilities—calculated as a function of the total fixed capital investment.

**TABLE 13.3 CoG Estimation**

Category	Description
A. Capital charge	From Table 13.2
B. Materials	
1. Process media	Cell culture media
2. Process buffers	Process solutions
3. Cleaning materials	Caustic (i.e., NaOH) and acids (i.e., H3PO4) for cleaning
C. Consumables	
1. Column resins	Protein A, ion exchange matrices
2. Disposable bags	Single-use plastic bags
3. Filters	Depth, ultrafiltration, and viral and sterile filters
D. Labor	
1. Process	Direct production labor costs
2. Quality	$v\%$ of D1
3. Others (e.g., logistics)	$w\%$ of D1
E. Others	
1. Insurance	$p\%$ of TCI
2. Engineering and spares	$\sigma\%$ of D1
3. Utilities	$\tau\%$ of TCI
Total	$A + B + C + D + E$

The factors used in the estimation can be obtained from benchmarking studies of biomanufacturing facility operations at a comparable scale.

*13.3.1.3.11 Output CoG.* The key cost components (i.e., capital charge, materials, consumables, labor, and other applicable costs) are summarized in Table 13.3, constituting the estimation of CoG. The cost of operation takes into account the impact of facility throughput, material costs, labor costs, etc. to provide a better approximation of the operating costs of the manufacturing process.

**13.3.2 Process Simulation for mAb Manufacture**

Traditional modeling methods include general-purpose simulators or spreadsheet-based evaluators, e.g., Microsoft Excel, equipped with numerical solving techniques, but these methods are often static and limited, leaving a gap between the industrial management and process systems. There are several process simulation software packages available commercially. However, only a few are directly applicable to bioprocessing. Three packages are described below.

BioProcess Simulator (BPS) (Aspen Technology, Inc., Cambridge, MA, USA) has been used to model industrial biotechnology processes. BPS, which is an extension of the established chemical process simulator Aspen Plus, is the first commercially available simulation tool for the biotechnology industry.

However, BPS has retained several chemical engineering characteristics that made it difficult to evaluate bioprocesses. The simulation tool was withdrawn in 1998 and was replaced by Batch Plus, a recipe-driven modeling platform that can perform simulations on multiple batches.

SuperPro Designer/SchedulePro (Intelligen, Inc., Scotch Plains, NJ, USA), a process simulator that can handle both batch and continuous processes, emerged on the market later. SuperPro Designer has its roots in BioPro Designer, the development of which was initiated at the Biotechnology Process Engineering Center of Massachusetts Institute of Technology to address the needs of the biopharmaceutical industries. SchedulePro is a separate package designed to work with SuperPro in order to look at the scheduling of multiple batches and associated support activities.

BPS Simulation (Biopharm Services Ltd., Chesham, UK) is a resource-constrained discrete event model. In this type of model, simulated time advances from one event to another and the time between events is unlikely to be equal. This simulation tool seeks to capture the distinct characteristics of biopharmaceutical manufacture, which comprises batch operations with complex scheduling for the main production run and support activities. A resource-constrained scheduler has been developed to generate the timing sequence of process operations and support activities in the simulation tool. The unique features of the tool include the ability to add multiple products in a single facility, to support resource-constrained allocation, to model ancillary activities [e.g., cleaning in place/steaming in place (CIP/SIP), buffer preparation, utility generation], and to implement work shift patterns. The integration of such features into a common platform is important to provide an actual representation of the entire biomanufacturing process.

Until recently, there have been a few publications to provide a critical assessment of these software packages. Rouf and colleagues (10) demonstrated the ability of Aspen BPS and SuperPro Designer to evaluate the production economics for the large-scale manufacture of tissue plasminogen activator from Chinese hamster ovary (CHO) cells. Aspen BPS was shown to be more geared toward chemical processes. As the calculation mode is rigorous, it requires more data to make appropriate use of the package. SuperPro Designer is more suitable in the absence of detailed data. In another study, researchers at University of Maryland, Baltimore, MD, USA carried out a comparative study between Aspen Batch Plus and SuperPro Designer to evaluate a vaccine manufacturing process under development at Merck & Company (11). These two simulation packages could successfully perform specific simulation tasks including the execution of basic material and energy balances, explore equipment change, and analyze process economics. However, such packages do not support dynamic resource allocation to monitor workloads. As the availability of resources is usually limited in the production facility, the deficiency to account for resource constraints could vitiate the accuracy of any model to provide an actual reflection of the entire manufacturing process. Since these reviews, some of the shortcomings of SuperPro have

been mitigated by the introduction of SchedulePro. This new extension allows multiple batches and associated support operations to be scheduled and overall resource utilizations to be predicted, although the results are still not based on a full resource-constrained discrete event simulation.

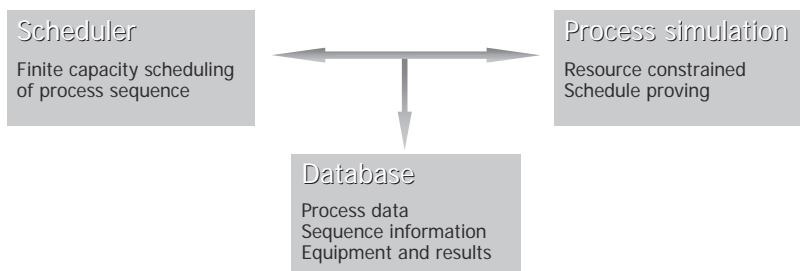
**13.3.2.1 Process/Facility Simulation.** Biopharm Services is a leading company in the design and development of advanced software models for the streamlining and optimization of biomanufacturing processes and systems. It has carried out simulation projects for a large number of organizations that include both engineering and operating companies. These projects require models that will simulate the workflow and all associated resource dependencies within the manufacturing environment. The facility's performance can be evaluated in simulated time within the model, allowing performance to be evaluated prior to the implementation of changes. Below is a list of typical areas where this type of process simulation is used.

- Reproduce an existing operations performance
  - Identify bottlenecks
  - Evaluate options for increasing capacity
  - Evaluate multiproduct production schedules
  - Predict the impact of a new product introduction or change in a process
  - Support logistics and provide cost insights into the operation
- Predict the performance of a new facility
  - Identify capital requirements
  - Assess the degree of flexibility
  - Optimize capital
  - Review the impact of future process improvements

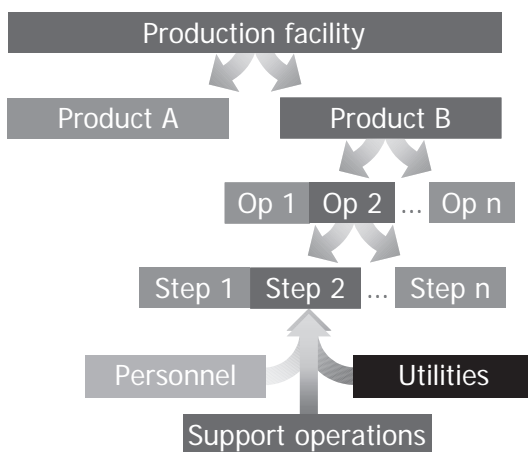
The BPS Simulation package is the basis for the following description of a dynamic process simulation platform. The models used for process simulation follow a common structure comprising a database, scheduler, and discrete simulation engine (Imaginethat Inc., San Jose, California, USA). Data flows are illustrated in Fig. 13.4.

**13.3.2.1.1 Database.** To represent the manufacturing processes, it is necessary to establish a database of information that defines the operations. The level of detail reflects how resources are allocated in a way that enables a “true” representation of the facility's dynamics. Figure 13.5 shows the hierarchical approach used to represent the manufacturing facilities. In essence, its products define a facility. The operations (Op 1, Op 2, etc.) and resources used to manufacture the products provide insight to the operations performance.

The data tables are divided into three basic components.



**FIGURE 13.4** Simulation structure.



**FIGURE 13.5** Data structure.

1. Process data—tables used to define the products and manufacturing sequences to be simulated
2. Support operations—tables that define the activities carried out in support of the main process operations
3. Resources—tables that define the resources, such as personnel and vessels, used by the process and support operations

**13.3.2.1.2 Process Simulation.** The workflow is reproduced in a discrete event modeling package provided by Imaginethat Inc. To develop models of this detail, the package should effectively replicate all related operations and their dependencies that define the manufacturing operation. A standard representation of batch workflow that addresses the specific requirements of biomanufacturing has been developed. This standard workflow representation allows the work center configuration defined in the discrete event modeling package to be defined by the database. This approach facilitates the addition

of resources and allows the simulation of multiple product manufacture without specialist simulation knowledge.

**13.3.2.1.3 Scheduler.** The final component is a multiproduct reactive scheduling tool that governs the unit operations within the model. The tool builds a process schedule based on a simple user-defined input sequence. The schedule can then be updated as the model runs to account for contamination events or delays associated with support activities.

The modeling approaches have been used in numerous operational facilities, and direct comparisons between the model output and facilities operation show good agreement (Fig. 13.3).

**13.3.2.2 Data Requirements.** To build the database, we have a well-defined data structure that must be populated with data sets that are described in more detail in the following sections. Not all listed data requirements would be needed for all projects.

**13.3.2.2.1 Process Information.** This describes the operations that must be performed and how they are related, durations, and resource requirements. It should include the main process flow plus all support systems, e.g., buffer preparation, cleaning, and sterilization regimes where they are not equipment specific.

- Process description
- Process flow diagrams
- Mass balance
- Process sequences
- Personnel allocation
- Exception handling data

**13.3.2.2.2 Equipment.** A definition of equipment requirements is used for manufacture. The selection of available equipment including the entire support infrastructure must be defined and included within the model, specifically,

- a list of equipment available within the facility, together with key sizing and relevant operational information; this could include storage areas for holding products and intermediates;
- materials of construction/surface finish constraints;
- specific requirements relating to allocation, sanitization, sterilization cleaning, etc.;
- other parameters that may have a bearing on availability (floor area requirements);
- scaling information/restrictions;
- failure behavior (sterility, equipment breakdown).

**13.3.2.2.3 Quality.** Sample processing and handling, including specific assays, should be included where they have a direct impact on the process operations, e.g., QC hold points, together with any steps that may have quality-associated rejection rates, and any specific requirements where there is a direct impact on the process/scheduling, etc.

**13.3.2.2.4 Materials/Consumables.** A list of materials and consumables where tracked must be defined within the modeling framework, in particular defining

- availability;
- storage constraints/shelf life;
- quality attributes and any specialist testing requirements.

**13.3.2.2.5 Operational Parameters.** The main parameters relate to specific operation requirements, such as shift patterns, and rules associated with scheduling of the operations.

**13.3.2.3 Bioprocess Simulation in Relation to ANSI/ISA–88.** The process of model build is similar to the development of process control software: it requires a data set that provides the basis for the simulation. In S88 terms, the model build requires as its input the general recipe and the facility information (physical model). Populating the database and configuring the discrete event model mimic the transformation from the general recipe to a master recipe. When a simulation is run, the model automatically generates the control recipe for each batch specified in the production schedule.

The models are not required to look at capacity resource profiling, etc., and as such they do not need to detail control phases and to model the individual components (such as valve actuation and control loops).

For these types of models, the information requirements are those for the operation of the manufacturing plant and its associated resources. The assumptions are that there is good good manufacturing practice (GMP) fit, that operators are trained, and that environmental and safety requirements are met. Hence, information regarding fine details of regulatory issues, testing, etc., is beyond the scope of most simulation projects. However, enough information must be retained in order to allow the process to be mapped to specific equipment within a facility.

## 13.4 COST MODELS IN PRACTICE

In recent years, cell culture titers have increased rapidly through improved expression/selection and media optimization. Modern processes expect to achieve titers in the range of 1–4 g/L with the prospect of meeting 10 g/L in



the next 5–10 years. Some companies are now quoting 20 g/L as their stretch target (12).

Within downstream processing, the improvements have been less significant and have been gained predominantly from incremental improvements in the capacity and robustness of chromatography resins. Some good examples of this include the MabSelect™ Protein A chromatography resins produced by GE Healthcare and the custom pseudoaffinity matrices offered by Prometics. The introduction of these products and an increasing emphasis on higher purification yields have led to the development of processes with fewer unit operations (see Chapters 11 and 12).

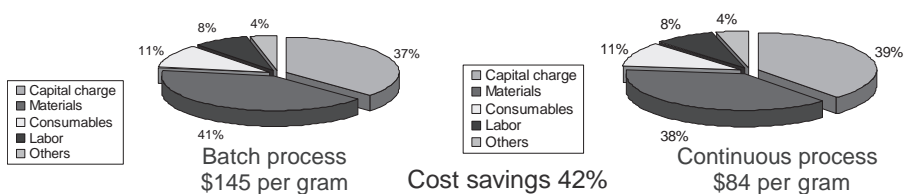
The growth in the number and sales of mAb products together with the high cost of manufacture has made the industry realize that manufacturing costs have to be reduced. The cost reductions being sought are significant and it is in this environment that cost modeling provides a rigorous method to evaluate process options and technologies, in particular to assess the cost benefits of the following at the unit operation level and at the factory level.

- New manufacturing technology (disposable chromatography, single-use bags, etc.)
- New operation methods (continuous upstream/downstream, method of buffer preparation, etc.)
- Process improvement and/or process options (crystallization, titer improvements, etc.)

### 13.4.1 Manufacturing Strategies—Disposables vs. Continuous

Continuous chromatography involves the simultaneous use of several columns. Simulated moving bed (SMB), the basic continuous chromatographic technology, simulates countercurrent contact between the solid and the mobile phases (see Chapter 17). There are several companies working in this area, including Bioflash and NovaSep. Continuous chromatography allows the whole of downstream processing to be run as a continuous operation maximizing the cost benefit. This is achieved by coupling it with continuous ultrafiltration, allowing the whole of the downstream process to be moved from batch to continuous processing.

To assess the potential of this approach compared to batch processing, a cost model was prepared by Biopharm Services based on NovaSep SAS technology. NovaSep SAS pioneered SMB technology in the pharmaceutical industry, with laboratory-scale systems in 1990 and the first industrial systems in 1997. Countercurrent flow allows a continuous flow of feed material to be separated, which improves the throughput and the utilization of the equipment compared to traditional batch chromatography. The cost models were used to evaluate the process economics of batch and continuous processing based on the production of a typical commercially relevant mAb process. A comparison



**FIGURE 13.6** Continuous vs. batch process economics.

was made between a batch and a continuous process running the same process at the same throughput. Both processes had the same upstream component. In the continuous model, all downstream unit operations ran continuously except the virus inactivation step, which was operated in a batch mode. Unit operations were connected by tubing, thereby eliminating the need for product hold vessels.

The main impact of continuous downstream processing was the smaller equipment and facility footprint for a given capacity. A potential capital reduction of 30% was seen compared to batch production, translating to reduced CoG. Figure 13.6 shows the breakdown of the cost categories for the batch and continuous processes. In both operational modes, the capital expenditure is the main cost contributor, followed by materials. By switching to continuous chromatography, most savings are gained in material costs (19%), followed by the capital charge (14%). The cost model reveals that the continuous process is more economically feasible compared with the batch process with a total operating cost reduction of about 42%.

### 13.4.2 Manufacturing Technologies—Single-Use Systems

Disposable technologies have been used in the industry for the last 15 years, but it is only within the last 7 years that they have gained widespread acceptance. The main application is solution handling, where most companies now use disposable containers for holding buffers, media, and products up to the maximum commercially available bag size (3000 L). Emerging areas that are gradually gaining acceptance include the following:

- Disposable bioreactors (up to 1000 L). These are now used in GMP production especially for seed preparation. We would expect their use to become more widespread within the next 5 years.
- Disposable mixing systems. The business case for disposable mixers has yet to be developed because of technical issues. Therefore, although there are already products on the market, they have yet to gain widespread acceptance and the costs are too high.
- Membrane chromatography (low capacity). This method is finding significant acceptance for the removal of impurities in flow-through mode for

the purification of mAbs (see Chapter 11). We predict it will find widespread use in these areas over the next 2–5 years.

- Membrane chromatography (high capacity). High-capacity membrane chromatography does not yet exist and technical challenges remain, though some companies (Sartorius, Pall, Bioflash, Upfront) are preparing capture columns in disposable formats. Cost is likely to be an issue here.
- Ultrafiltration systems. Currently, these are only available for small-scale use and there are technical problems to do with scale-up, so we do not envisage widespread use for medium- to large-scale processing.

The emerging field of disposable technologies has the potential to reduce initial start-up capital costs and plant complexity significantly, and may play an increasing role in biomanufacturing operations—perhaps more efficiently than their reusable counterparts—without sacrificing product quality. The traditional stainless steel equipment would be replaced by presterilized disposable components incurring minimal cleaning costs. In addition, such technologies offer greater process flexibility; they simplify material and personnel flow, eliminate cleaning validation costs, and reduce the risk of cross contamination between batches in multiproduct facilities. Disposable-based engineering could be implemented throughout the production plant (13), such as the use of single-use membrane chromatography devices, disposable prepacked chromatography columns, and single-use bags for fluid handling. A detailed study of the design concept for a facility based around single-use systems and the potential benefits of such a layout has been carried out (14, 15).

**13.4.2.1 Impact on Product and Solution Handling.** The process used for this assessment is based on the production process for a typical mAb (Table 13.4). This reference process is selected according to commercial relevance and sourced from details that are available in the public domain. The process consists of the inoculation of the seed fermenter through to bulk-purified sterile-filtered product.

To assess the impact of disposable solution handling technologies, BioPharm Services prepared a cost model based on the typical mAb process operated at  $2 \times 5000$  L production scale with a product titer of 2 g/L. The main difference between the two manufacturing options is that one uses stainless steel vessels and the other implements the use of disposable bags for fluid handling. The process steps and all the associated major equipment are similar in the cost model. The estimates for the cost parameters are drawn from data contained in BioPharm Services' proprietary cost databases consisting of benchmarking information from over 10 biomanufacturing operations.

The vessels and bag containers are classified according to specific duty in the facility.

- Product hold
- Media preparation

**TABLE 13.4 Process Sequence**

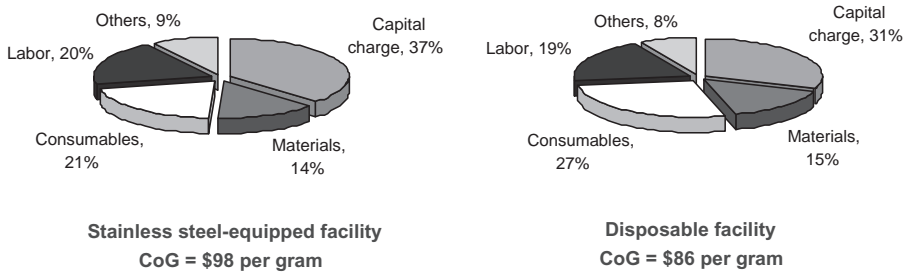
No	Category	Unit Operation	Yield, %	Product		
				Conc, g/L	Volume, L	Mass, g
1	Cell culture	50-L seed bioreactor				
2	Cell culture	500-L seed bioreactor				
3	Cell culture	5000-L production bioreactor		2.00	5,000	10,000
4	Recovery	Centrifugation	85	2.00	4,250	8,500
5	Recovery	Depth filtration	85	1.70	4,250	7,225
6	Purification	UF/DF #1	95	16.15	425	6,864
7	Purification	Affinity chromatography	90	3.28	1,885	6,177
8	Purification	Virus inactivation	98	3.15	1,923	6,054
9	Purification	Ion—exchange chromatography	95	6.10	942	5,751
10	Purification	Polishing chromatography	95	5.80	942	5,464
11	Purification	Viral filtration	98	5.68	942	5,354
12	Purification	UF/DF #2	98	27.84	188	5,247
13	Purification	Sterile filtration	98	27.28	188	5,142
<b>Overall downstream process yield</b>			51			

- Media hold
- Buffer preparation
- Buffer hold

The cleaning of vessels is a major user of quality utilities in the biotechnology facility. Cleaning is required after every vessel use. In the disposable option, single-use bag systems are used to prepare and store media, buffers, and products prior to further processing within the facility. The bag systems are provided preassembled, sterile and ready for process use. Vessel liners are used to hold the bag for solution preparation. Where the solution volume exceeds the maximum bag size, a stainless steel vessel of the next available size is selected. The limitations of the bag technology in the facility are

- maximum single-use hold bag volume of 3000 L;
- solution preparation in disposable bags up to 2000 L.

Each solution prepared in-house is passed through a 0.2- $\mu$ m filter prior to storage in a hold tank. The disposable hold bag comes with a sterile filter. The cost analysis in this section identifies differences (i.e., water usage, capital requirements, fixed and variable operating costs) in the performance of the



**FIGURE 13.7** Breakdown of CoG for the two manufacturing options.

disposable bag production line when compared to the conventional stainless steel vessel facility.

**13.4.2.1.1 Results.** There was a 26% reduction in capital for the disposable-based facility where the capital fell from \$67 million to \$49 million. To gain insight into the impact of disposables, the CoG was analyzed by comparing the impact of this technology on capital charges, materials, consumables, and labor.

A summary of the CoG breakdown is provided in Fig. 13.7. As expected, the costs of some categories (e.g., cell culture media, process buffers, column resins) are the same for both options as the manufacturing strategies differ only in the preparation and holding of fluids. The key outcomes are the following:

- The capital charge is the highest cost-saving category. The reduction in the process equipment minimizes the extent of the design and installation, which significantly reduces the capital requirements.
- The labor category contributes about 3% to the total cost savings. This is attributed to the reduction in vessel cleaning activities.
- Material savings of 1% are gained by reducing the consumption of caustics and acids used for cleaning.
- There is an overall 4% increase in the consumables category due to the use of plastic disposable bags.
- The cost benefits provided by reduced process equipment, material consumption, and labor more than compensate for the increased consumables costs, translating to an overall CoG saving of about 12% in the running costs per gram for the disposable option.

By considering all aspects of operation, including capital investment, materials, consumables and labor, the key cost benefits of single-use technology can be identified.

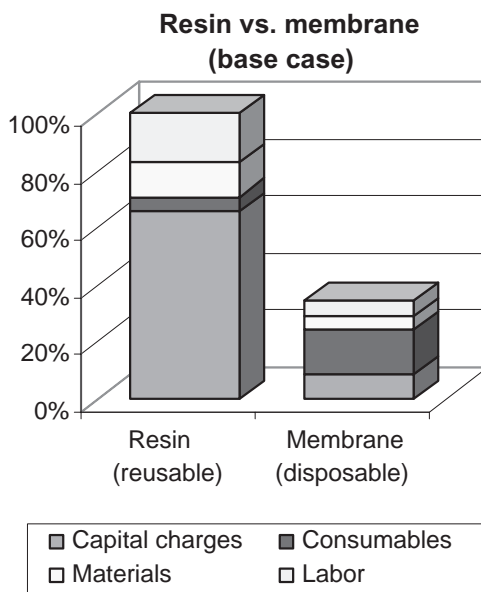
- Disposables switch fixed capital costs to variable consumables costs that are only applicable when the facility is operational.

- Overall operating costs for the disposable option are reduced despite the increase in consumables costs.
- Other disposable benefits such as the simplification of materials and personnel flow, the streamlining of process development, and the elimination of cleaning validation cannot easily be quantified. These benefits increase the attractiveness of this technology.

**13.4.2.2 Membrane Adsorbers.** Single-use technologies can also be used for downstream processing, and we now consider the impact of disposable membrane absorbers applied to flow-through chromatography, which displays this technology to maximum advantage. Flow-through mode (i.e., the matrix binds the contaminants and not the product) is used in mAb manufacturing for the removal of impurities and viruses during late-stage purification. In mAb manufacturing, the absolute amount of impurities is small and as a consequence, the chromatography system is sized according to flow rate as well as quantity of feed.

In the example case, Biopharm Services prepared a cost model to provide an economic comparison of the conventional flow-through resin-based chromatography column and a disposable membrane chromatographic device (a cost mode based on Sartobind Membrane Adsorbers) (16).

Figure 13.8 shows the cost comparison of both chromatographic methods in flow-through mode. Again the main impact of the disposable membrane



**FIGURE 13.8** Breakdown of cost categories for the reusable resin-based chromatography and the disposable membrane chromatography.

technology is reflected in the amount of equipment required for the unit operation. The model indicates cost savings in the capital charge (57%), material consumption (7%), and labor (12%). However, there is an increase of 11% in consumables costs largely reflecting the use of membrane capsules, which are discarded after each chromatography step. The benefits of reduced process equipment, material consumption, and labor more than compensate for the increased cost of membrane chromatography media, translating to a 66% reduction in CoG. The disposable option has the advantage of switching capital costs to consumables and thus from fixed costs to variable costs that are only relevant when the plant is operational.

### 13.5 SIMULATION IN PRACTICE

There is an increased awareness within the industry that manufacturing is a core competence and that operational excellence is required. With this drive for operational excellence, the industry is finding that simulation is necessary to analyze and optimize complex manufacturing processes. Historically, simulation has been used by engineering companies on new capital projects as a design tool to optimize capital requirements. It is now understood that simulation tools have wider applications, beyond capital optimization and through into facility start-up and operation. Simulation tools are used to support some of the initiatives described below.

- Benchmarking
- Managing change—multiproduct manufacture, changeover, process improvements
- Resource management—people, equipment, shift patterns
- Optimizing capital infrastructure—solution preparation, high-quality utilities
- Facility start-up
- Logistics—buffer and media management, warehousing, supply chain
- Supporting operational effectiveness initiatives

In this section, two case studies will be used to illustrate how simulation is used today.

#### 13.5.1 Managing Multiproduct Manufacture

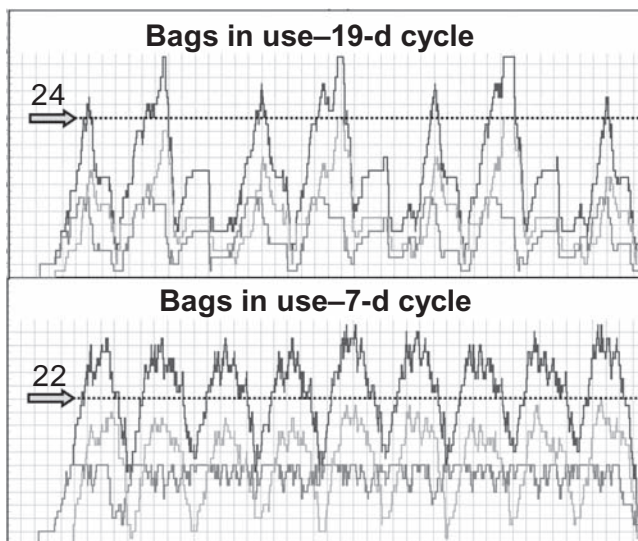
Contract manufacturing organizations (CMOs) manufacture products for third parties and have to ensure their facilities are highly utilized in order to remain profitable. Therefore, they need expertise in the management of product introductions, product changeovers, and effective operations.

This example is based on a large-scale CMO. They focus on the manufacture of therapeutic proteins for clinical supply through to in-market supply. They

had recently expanded their manufacturing capacity introducing two microbial manufacturing streams to their site. The objectives of the process simulation prepared by Biopharm Services were to optimize the manufacturing operations and to support new product introductions as a part of the planning function. The model was designed to allow users to

- add new products to the model; this can be carried out by any process engineer trained in the use of the Excel database;
- evaluate the impact of new products in terms of capacity and its impact on operations (including manpower, utility infrastructure, etc.);
- assess the impact of production in one production line on another;
- optimize logistic issues such as shift patterns, planning, and management of buffer preparation and media preparation.

The process simulation was set up and validated and the outputs compared to the start-up product runs carried out in the facility. Once validated, the simulation was used to evaluate the best methods required to maximize production within the facility. One of the aspects considered was to understand and optimize the buffer supply to the downstream purification area. Buffers are held in 1000-L disposable hold bags, and the issue that had to be addressed was how to manage the size of the buffer inventory as the production rate was scaled up. In Fig. 13.9, the upper profile shows how the inventory of buffer bags varies with time for a production pattern where harvest occurs from two



**FIGURE 13.9** Buffer container inventories as a function of time.



bioreactors every 19 d, the problem being that when the inventory reaches 28 bags, the plant has no more capacity to accommodate buffer containers. The model was used to analyze the buffer supply chain and to optimize the methods used to manufacture and order buffers. The outcome is shown in the lower chart of Fig. 13.9 where the production rate has increased to two bioreactor harvests every 7 d (a 2.7-fold increase in capacity) while keeping the inventory to about 28 bags.

Model users have successfully deployed discrete event dynamic modeling to identify productivity improvement targets, prioritize capital improvements, and support new product introductions. By using the models in this way as they introduce new products, they are reducing risk by gaining insight into people and plant constraints for the different processes. To the user, simulation provides

- insight into the complexities of biotechnology plants;
- help with setting direction in projects and improvement programs.

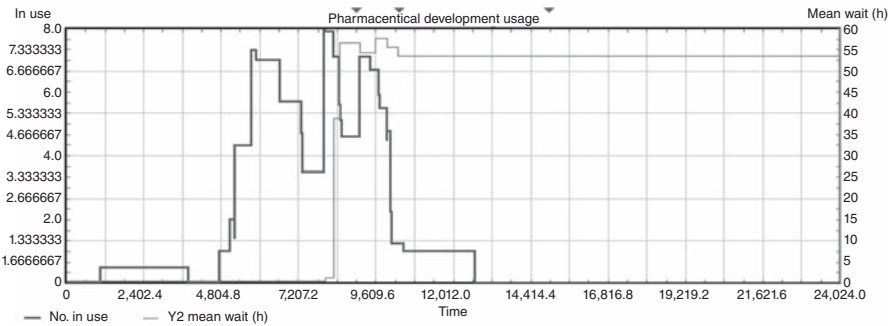
### 13.5.2 Resource Management

In this second case study, a medium-sized biotechnology company has a growing portfolio of development projects and expects activity levels in both development and GMP manufacturing to increase significantly over the next 5 years. The company wanted a model of its operations to assess the impact of these increases in terms of resources such as people and equipment and to understand their capacity. In this case, they wanted a discrete event simulation to encompass their whole operation, covering development laboratories, pilot plant, and the GMP manufacturing operations. This allowed them to understand the interactions and facilities in terms of personnel and relative timings.

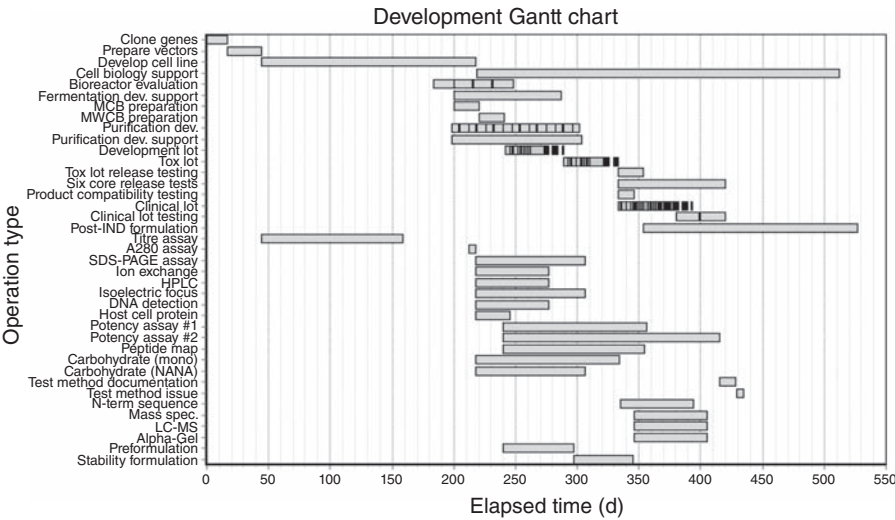
The company wanted a data-driven method to answer several questions.

- How do we maximize the use of development capability without overextending?
- How do we get more productivity from existing resources?
- What productivity gains are possible if new resources are added?
- What impact will development projects have on the utilization of pilot plant and GMP manufacturing facilities?

The unified model delivered to the company allows users to define new development and manufacturing projects as required. Each project is defined as a series of linked development or manufacturing sequences. Each sequence can include any number of associated tasks, such as a particular manufacturing operation or assay development activities. The overall project plan is specified by the user via simple scheduling constraints, e.g., specifying that a



**FIGURE 13.10** Personnel profile for a pharmaceutical development group (one project).



**FIGURE 13.11** Gantt chart for a development project.

development lot cannot be manufactured until the master cell banking task is completed.

The simulation model then uses this information to build an initial schedule to drive both development and manufacturing activities that draw on constrained resources such as equipment, personnel (Fig. 13.10), and materials over the course of the project time line (Fig. 13.11). The linkages between different phases of the projects allow any delays to propagate naturally. For example, a contamination event during the manufacturing of a development lot will delay subsequent lot testing and clinical material manufacture.

Once the model was delivered to the client, a number of key users were trained to populate the database. Following the training, predictions from the

integrated development model were validated using information about current and historical development projects. Once the client was comfortable with the database and its output, the model was then incorporated into their business development process to support a number of key decisions with a typical investment of only 2–3 h required to define each new project.

- Can a new project be incorporated into the existing schedule?
- What impact will the new project have on existing activities, if any?
- Can we assure a partner company that we have sufficient capacity to meet their requirements?
- Is additional equipment or headcount required, and if so when?
- What resources are required to complete a project as soon as possible?
- What resources are required to meet a specific project completion date?

### 13.6 ACKNOWLEDGMENTS

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# 14

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## **ALTERNATIVES TO PACKED-BED CHROMATOGRAPHY FOR ANTIBODY EXTRACTION AND PURIFICATION**

JÖRG THÖMMES AND UWE GOTTSCHALK

### **14.1 INTRODUCTION**

The titer of monoclonal antibodies (mAbs) produced by cultured mammalian cells has increased dramatically since the first commercial production began over 20 years ago. It is now possible to achieve titers of 10 g/L for some mAbs and there are no signs of this trend slowing down, making 20 g/L titers a realistic projection for mAb production in the next decade (1). As the productivity of upstream processes continues to increase, the question arises as to whether the productivity of the subsequent purification steps will be sufficient to keep pace. It has been suggested that conventional purification processes may be overwhelmed by the sheer amount of protein being delivered to downstream operations (2). Additionally, it may be argued that the increase in bioreactor productivity will also result in a substantial increase in the amount of impurities to be removed by purification steps, which in turn might overtax the ability of conventional steps to resolve large amounts of target protein from similarly large amounts of impurities. In this context, difficulties in scaling up chromatographic separations have been discussed (3). While the theory of the “purification bottleneck” has been well covered recently, alternative opinions have also been published, making the case that conventional purification technologies could support the production of biologics up to the ton scale (4).

While the jury is still out on the fate of conventional purification, and the outcome might even vary on a case-by-case basis, it is nevertheless valuable to discuss alternatives to conventional purification technology. When looking for alternatives to existing technology, two approaches may be pursued. First, the development of new, high-technology solutions may overcome perceived bottlenecks in downstream processing by increasing the capacity and throughput of current chromatographic separations. Second, the implementation of inexpensive solutions that have been used successfully in lower-margin industries (e.g., the food and detergent industries) may replace chromatography or may at least take some of the pressure off chromatography steps allowing the entire process train to be simplified (5). Some of the most promising high-technology solutions are discussed in detail in other chapters, e.g., simulated moving bed chromatography (see Chapter 11), large-scale precipitation of impurities (see Chapter 15), and the use of charged filtration membranes (see Chapter 16). In this chapter, we focus on some of the solutions that are already applied successfully for the purification of conventional pharmaceutical entities, which are now being investigated with increasing enthusiasm for the production of biopharmaceuticals, including mAbs.

## **14.2 INCREASING THE SELECTIVITY OF HARVEST PROCEDURES: FLOCCULATION OF PARTICULATE AND NONPARTICULATE IMPURITIES**

The first series of operations in a purification train—the harvest step—focuses on the removal of particulate impurities with the goal of delivering a particle-free supernatant that can be fed into the subsequent, mostly chromatography-based, processes. Typically, tangential flow microfiltration, depth filtration, and in particular centrifugation are used to clarify cell culture suspensions. These initial steps remove larger particles ( $>1\text{--}2\mu\text{m}$  diameter) and are frequently followed by the use of a polishing depth filter and one or more absolute filters to remove smaller particles and to reduce the turbidity of the feedstream. High-titer cell culture processes are deemed to be more difficult to harvest, mainly due to the fact that higher bioreactor productivity is in part accomplished by increases in cell density and longer process duration, which may reduce cell viability. The resulting suspension is characterized by higher particle content (more than 5% wet cell mass has been reported) as well as a wider distribution of particle sizes, and by a greater number of small particles. This complicates the clarification process, so membranes are increasingly being replaced by centrifugation to overcome the problem of reduced product transmission through fouled membranes.

One approach to deal with the increase in the concentration of small (sub-micrometer) particles is the use of flocculants to create larger particles, which are easier to separate from the cell culture fluid. Flocculation is a similar process to coagulation, where suspended particles clump together because the

attractive forces between them overcome any repulsive forces caused by like surface charges. Such repulsive forces can be eliminated, e.g., through the addition of inorganic electrolytes, which shield the surface charges, or by the addition of polyelectrolytes that bind to and neutralize the surface charge (6, 7). Flocculation is the agglomeration of particles caused by the bridging effect exerted by polymers that are adsorbed to more than one particle. It has been used mainly for the removal of whole cells from fermentation broth, and more recently for the removal of cell debris and proteins. Particles are often coagulated or flocculated prior to filtration and/or centrifugation to reduce the passage of small noncoagulated particles through the filter and to produce a more porous cake that is easier to remove.

Often, polymers such as polyethyleneimine are added to the solution to induce flocculation and to enhance the removal of cellular debris by centrifugation, although nontoxic flocculation agents such as calcium chloride and potassium phosphate avoid toxicity and removal issues caused by the use of polymers (6, 7). By adding 30 mM calcium chloride and then 20 mM potassium phosphate, a calcium phosphate precipitate forms rapidly and flocculates cellular debris. Within minutes, a clear supernatant layer begins to form during gravity sedimentation. Centrifugation for 10 min at  $340 \times g$  yields a clear supernatant and approximately 95% recovery of mAb.

In addition to simplifying the harvest process, flocculation can also contribute to the clearance of soluble impurities. It has been shown that reducing the pH during harvesting can induce the precipitation of process-related impurities such as host cell proteins (HCPs) and DNA (8). Depending on pH, the DNA concentration can be reduced to below the detection limit and HCP can be reduced by up to 80%. Adding selectivity to the harvest process in this manner can therefore reduce the overall impurity load in downstream purification steps. This has several benefits, including a better product-to-impurities ratio (allowing the load on downstream chromatography steps to be increased, thus shifting the focus from high resolution to high capacity) and clearing the feedstream (so increasing the number of cycles achieved during the useful lifetime of the resin). In the most ideal case, removing an impurity upfront may reduce the number of chromatographic separation steps required.

## **14.3 SOLUTIONS FOR ANTIBODY EXTRACTION, CONCENTRATION, AND PURIFICATION**

### **14.3.1 Extraction and Concentration by Precipitation**

Extraction refers to any process where one or more components of a mixture are transferred from one phase to another, or where such components are removed from the mixture all together. Precipitation is therefore a specific form of extraction that involves the removal of one or more soluble components from solution to form a solid phase (the precipitate). Precipitation is

among the simplest and least expensive fractionation methods since it can be achieved by simple changes in solution conditions, e.g., the addition or removal of salt and organic solvents, or even by changes in temperature and pH (9, 10).

Under mild conditions, protein precipitation is reversible and subsequent redissolution can restore total activity. Therefore, while precipitation can be used to remove impurities, whose subsequent activity is immaterial (11) (see Chapter 15), it can also be used to isolate the target protein in a mixture, in this case a mAb (12). In the former case, the differential solubility of proteins may be used for fractionation, or to remove bulk proteins with different solubilities to the target mAb, the resulting precipitate then being treated as a particulate contaminant for subsequent removal by centrifugation or by filtration, as would be the case with flocculation (see Section 14.2). In the latter case, precipitation and subsequent redissolution in a smaller volume of buffer not only reduces the processing volume, but the resulting solution also contains mostly dissolved protein, free from other soluble contaminants. Several groups have developed methods to precipitate mAbs in large-scale processes, and this could replace Protein A chromatography in the long term (13–15). Proteins can be precipitated by changing the pH or temperature or by adding a mild organic solvent, salt, a multivalent metal ion, or a nonionic polymer. The precipitation method chosen depends on whether the protein precipitate that is formed can be redissolved without loss of activity, the expense of the precipitating agent and its recovery, and the effects of precipitating agent impurities in the precipitate. In large-scale processes, the cost of the precipitant as well as the environmental impact of its disposal would be an important factor in determining its suitability.

One of the most widely used methods for protein precipitation is salting out. High salt concentration promotes protein aggregation and precipitation. Although the mechanism is not well understood, the salt is thought to remove the water of solution from the protein, thereby reducing its solubility. Different ions have different impacts on the solubility of proteins, and the effectiveness of these ions is described by the so-called Hofmeister series (16), with citrate as the most effective followed by phosphate, sulfate, acetate, chloride, nitrate, and thiocyanate. The salts at the low end of this series tend to cause structural damage to proteins. The high solubility of ammonium sulfate in water and the position of sulfate in the Hofmeister series make it the most popular choice for salting out proteins including mAbs as first demonstrated by Brieger and Ehrlich (17). Most antibodies precipitate at ammonium sulfate concentrations above 50% saturation. The salting out of immunoglobulins with the neutral salts ammonium sulfate and sodium sulfate combined with the acridine dye Rivanol<sup>®</sup> was discussed by Heide and Schwick (18).

Adjusting the pH is also widely used to precipitate proteins, since proteins are soluble in water due to the interaction of their charged groups with ionized water molecules. Adjusting the pH to the isoelectric point (pI) of the target protein reduces solubility to its lowest level, since the net charge of the protein



is eliminated. Most proteins have a  $pI < 7$ , and the relatively low cost of acids makes pH adjustment with acid a popular method of protein precipitation. However, too much acid or base can cause irreversible denaturation.

Metal ions such as  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ , and  $Ag^+$  bind to different protein functional groups and can cause them to precipitate. They act at much lower concentrations than the ions of the Hofmeister series and are easily removed by ion-exchange adsorption or chelating agents. Organic solvents can also cause protein precipitation. The addition of a mild organic solvent to an aqueous protein solution reduces the solvent dielectric constant, thereby inducing protein precipitation, as long as the solvent is completely miscible with water (e.g., ethanol or acetone). For example, the use of ethanol to precipitate proteins at their  $pI$ s in the large-scale production of blood plasma products was developed by Cohn and colleagues (19). Solvent precipitation is typically performed at low temperature ( $<10^\circ C$ ) because conformational rigidity then prevents irreversible denaturation. If the target protein is particularly resistant to denaturation, then high temperatures can be used to selectively precipitate other proteins by causing widespread denaturation, leaving the target protein in solution. However, this method is not used with antibodies, which tend to denature at high temperatures.

Polymers and polyelectrolytes can also induce precipitation (20). Nonionic, water-soluble polymers induce protein precipitation by excluding water from the solvation structure of a protein. Polyethylene glycol (PEG) is the most widely studied and widely used polymer, but dextrans are also used for this purpose. High PEG concentrations are required to precipitate low-molecular-weight (LMW) proteins, whereas low concentrations are required for high-molecular-weight (HMW) proteins. Polyelectrolytes such as polyacrylic acid, carboxymethyl cellulose and polyethyleneimines precipitate proteins at a much lower concentration (usually  $<0.1\%$ ) than nonionic polymers. They act more like flocculants (see Section 14.2) and adsorb to the protein. Thus, polyelectrolytes, unlike PEG, coprecipitate with the protein and can cause irreversible denaturation.

Early studies of precipitating agents included the investigation of short-chain fatty acids, particularly caprylic acid (*n*-octanoic acid) (21). Caprylic acid is only useful for processes in which the target protein remains in the supernatant because protein–fatty acid complexes formed in the precipitate are difficult to dissociate. After many years without an industrial application, possibly because caprylic acid is a poor target protein precipitant, it is now used to remove contaminating proteins in the industrial-scale process for two IgG products (22, 23). Several groups have also shown the ability of caprylic acid to inactivate enveloped viruses (23, 24).

### 14.3.2 Extraction and Concentration by Liquid-Phase Partitioning

Liquid–liquid extraction using organic and aqueous extraction media is a traditional separation operation that has been applied to the purification of many

different types of protein (25, 26) including mAbs (27). In three-phase partitioning (TPP), proteins can be purified directly from cell homogenates by partitioning between a layer of butanol and a strong aqueous salt solution. Under these conditions, cell debris tend to separate into the organic phase and nucleic acids precipitate at the interphase, while proteins remain in solution. The selectivity of extraction can be predicted by mathematical modeling (28) and through the inclusion of affinity reagents such as metal ions in the system (29).

Aqueous two-phase systems are the most widely used extraction operations, employing a mixture of aqueous polymers and/or salts (30). One phase generally contains PEG and the other contains a different polymer, such as dextran, or salts. Under ideal conditions, the desired mAb can be separated into the PEG phase while the majority of contaminating proteins as well as other contaminants are trapped in the second phase, or in the interphase, and can be removed by centrifugation. Recent developments in aqueous two-phase extraction (ATPE) have been reviewed by Banik and colleagues (31).

### **14.3.3 Concentration by Evaporation**

In some biomanufacturing processes, evaporation may be a suitable volume-reduction step, using vacuum evaporation to minimize protein denaturation and operating at temperatures  $<40^{\circ}\text{C}$ . Foaming often causes equipment fouling and protein denaturation at the air–water interface. Ultrafiltration is a much gentler method of volume reduction for proteins and has largely replaced evaporation in industrial-scale applications (see Chapter 16).

## **14.4 NONCHROMATOGRAPHIC SOLUTIONS FOR ANTIBODY PURIFICATION AND FORMULATION**

### **14.4.1 Crystallization**

Crystallization is the separation of a solute from a supersaturated solution achieved by encouraging the formation of small clusters of solute molecules, which then grow into crystals. The crystallization process involves the formation of a regularly structured solid phase, which impedes the incorporation of contaminants or solvent molecules, and therefore yields products of exceptional purity (32). It is this purity that makes crystallization particularly suitable for the preparation of pharmaceutical proteins, coupled with the realization that protein crystals enhance protein stability and provide a useful vehicle for drug delivery, as has been demonstrated with various protein drugs including mAbs (33). Protein crystallization has been developed into a commercial technology for drug stabilization and delivery [e.g., (34, 35)]. Several manufacturing processes involve crystallization including recombinant insulin, aprotinin (36), and Apo2L (37).

The advantages of crystallization as a final purification and concentration step in clinical manufacturing processes include the following:

- Large-scale crystallization can replace some of the more expensive purification steps in the manufacturing process, making the whole process more affordable.
- Because reactions proceed very slowly if at all in the crystalline state, interactions between molecules are slowed down significantly, making crystals an ideal vehicle to store and administer mixtures of biological macromolecules.
- Solid crystalline preparations can easily be reconstituted into very highly concentrated formulations for injection, which is particularly useful when intended for subcutaneous administration.
- Protein crystals may be used as a basis for slow release formulations *in vivo*. Characteristics of the crystal such as size and shape, degree of cross-linking, and the presence of excipients can be manipulated so as to control the release rate.

There are numerous crystallization methods, including evaporative, cooling, precipitation, melt, and supercritical crystallization, each with specific processing methods and apparatus requirements. Evaporative precipitation and cooling crystallization are the most suitable for highly soluble products, including mAbs. However, the large-scale crystallization of proteins is challenging because protein molecules are large, and sometimes easily degradable, so they require carefully designed processes (38). In the case of mAbs, success with crystallization has been driven more by a desire to increase stability and to provide a delivery vehicle than by the desire to replace chromatography during downstream processing. Therefore, the results, while impressive, have little relevance to the industry as a whole at the current time. Challenges and limitations include process control, yield, buffer conditions (particularly whether buffers are compatible with native proteins), and the transfer of crystallization conditions to the crude feedstream (fermenter off-load). For example, it has been possible to generate 100- $\mu$ m crystals of a mAb in 3 weeks from a solution of 11% PEG 1000, 10.5 g/L mAb, pH 6.0 at 30°C, but the resulting mAb had a pI of approximately 4.7, much lower than most commercial mAb therapeutics (39). In addition, the phase diagram for a number of antibodies shows very few opportunities to find a crystallization window, most likely due to the high structural flexibility of the antibodies (40).

Crystallization is driven by supersaturation, which may be achieved by cooling, by evaporation of solvent, or by mixing two reactants or solvents. In all these cases, the actual concentration of the target molecule becomes higher than the equilibrium concentration, and a driving force for crystallization is achieved. Crystallization begins with nucleation, and nuclei then grow by a combination of layered solute deposition and agglomeration caused by random

collision. Crystal growth is opposed by the continual dissolution of the solid phase, but conditions are chosen that favor growth over dissolution. The interplay of all these processes determines the crystal size distribution (CSD) of the solid, which is an important component of the product specification since it determines the separability of particles, and how they respond to washing and drying. Because this is such an important property, the kinetic processes underlying crystallization have been extensively modeled, and can be predicted by using the population balance equation (PBE), which describes how the size distribution develops in time as a result of various kinetic processes.

Crystallization can be triggered in several ways, and these are divided into primary and secondary nucleation mechanisms (32). In primary nucleation, the solid phase forms spontaneously from the clear liquor. In cases of heterogeneous nucleation, crystals nucleate around tiny contaminating particles that are present in the solution. In perfectly pure liquor, there is no such substrate, and clusters of solute molecules are thought to form randomly, simply through the statistical fluctuation in their distribution (homogeneous nucleation). Secondary nucleation occurs where crystallization is already in progress. It reflects the formation of tiny crystal fragments through the collision of existing crystals with each other, or with the walls of the crystallizer, which then serve as nuclei for the growth of new crystals. Most crystals are formed by primary nucleation during the initial phase of evaporative or cooling crystallization. When these have grown to form larger crystals, secondary nucleation becomes the predominant source of new crystals. Once nuclei have formed, crystal growth can be uniform (deposition of molecules from the solute onto existing crystals) or nonuniform (agglomeration).

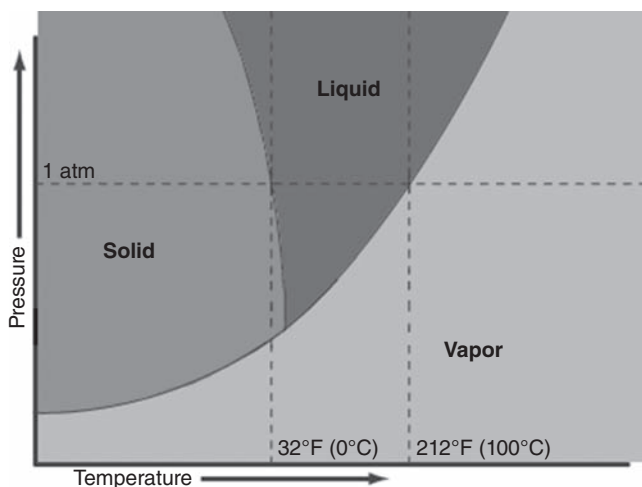
#### **14.4.2 Controlled Freeze–Thaw**

An often overlooked aspect of processing is the consequence of freezing and thawing biological materials. Conventional freeze–thaw techniques at a production scale can introduce challenges to the manufacturing process because this affects the distribution of protein and solutes in freeze–thaw containers. Where frozen solutions have been dissected and analyzed in detail, up to a 10-fold difference in the protein concentration has been observed in different parts of the container, depending on the freezing method and the scale. For this reason, companies such as Integrated Biosystems (now part of Sartorius Stedim) have developed proprietary controlled freeze–thaw systems specifically for the manufacture of biological products (41, 42). These are based on specially designed bags and freeze/thaw instruments that avoid concentration heterogeneity, giving an extended shelf life, improved reproducibility, batch homogeneity, and increased yields (43). Freeze–thaw products provide a controlled, validated way to freeze and then thaw biopharmaceuticals in a consistent and reproducible manner for developing, storing, or shipping the material. The technology also allows manufacturing steps to take place in different geographical locations if necessary (44).

### 14.4.3 Lyophilization

Lyophilization or freeze drying in pharmaceutical manufacture is a process in which a pure dissolved product is frozen and then dried by exposure to conditions that cause sublimation of the ice (45). With pure water as the solvent, reducing the pressure to less than 0.6 atmospheres in a vacuum chamber is sufficient to prevent the formation of liquid water when the frozen product is heated above freezing point (Fig. 14.1). The general approach is therefore to freeze the product and to place it on a heated shelf in a vacuum chamber. Once the chamber is evacuated to below 0.6 atmospheres, the temperature is increased to just above the freezing point of water resulting in the sublimation of the ice. At this stage, it is the bulk solvent that sublimates, and this may represent 10–100 times the volume of the dry product depending on its initial concentration. This process is known as main drying. Freezing and main drying are usually followed by a secondary drying step involving desorption of water that is bound to the solid. For pharmaceutical proteins, this means water molecules attached to the protein via hydrogen bonds. Such water molecules form a monolayer around the protein and have distinct properties to the bulk solvent. They may constitute as little as 5%–10% of the volume of the dried product, but even this amount of water can facilitate some enzyme-catalyzed reactions that would cause protein degradation. Once this water has been removed, the lyophilized solid is packaged under vacuum to prevent any further exposure to water until ready for formulation.

Lyophilization, like crystallization, is a useful final-stage procedure in biomanufacturing because it provides a way to prevent the reactions that



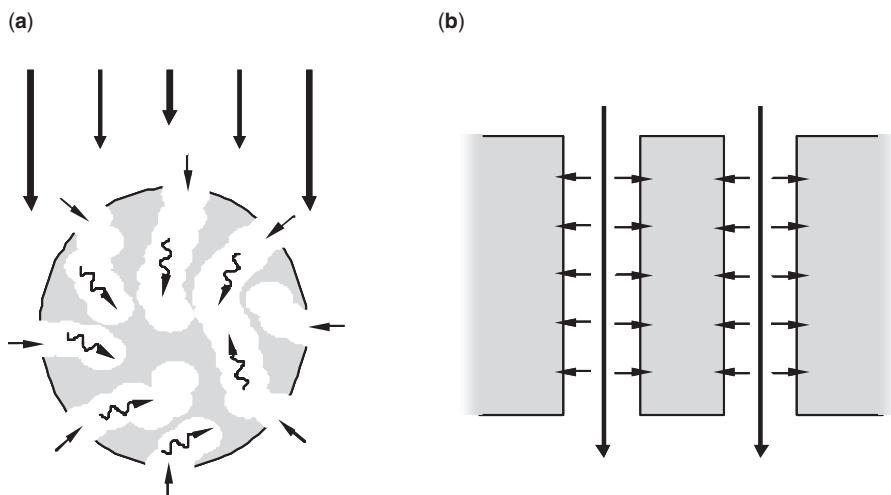
**FIGURE 14.1** A phase diagram for water, showing that sublimation occurs near the freezing point of water when pressure is reduced below 0.6 atmospheres.

normally occur in solution. Therefore, the process can enhance the stability of a protein pharmaceutical and can allow it to be stored for prolonged periods at ambient temperatures without fear of degradation or loss of activity. Lyophilization is relatively expensive to carry out for large-scale processes, but the benefits of increasing drug longevity and eliminating the requirement for a cold chain can far outweigh the initial costs.

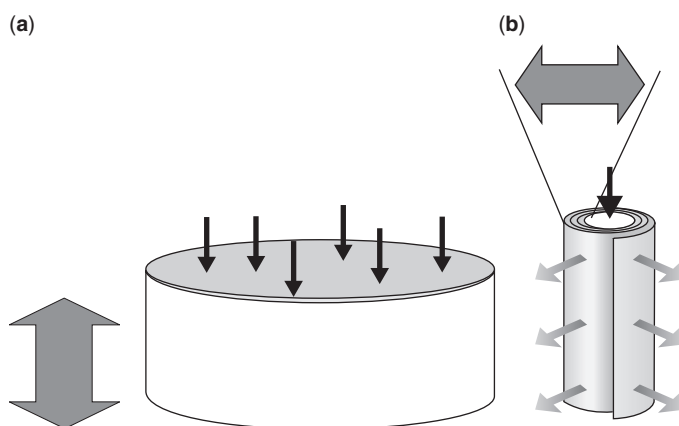
For pharmaceutical products, the lyophilization process should not impede reconstitution of the drug for formulation. For other products, such as cells, it is necessary to use cryoprotectants to prevent damage to cellular structures and membranes during freezing. For protein drugs, however, the tendency of nascent ice crystals to form pores in the solid product is actually beneficial, since this facilitates access for water molecules when the product is reconstituted and allows it to dissolve rapidly. This is important to maintain the product's structural integrity and biological activity.

## 14.5 MEMBRANE ADSORBERS

Membranes are integral to many bioprocesses because they can be used as disposable modules, but thus far, their principal application has been filtration rather than chromatography (see Chapter 16). Interest in membrane chromatography is growing because disposable membrane filters have reduced cleaning and validation costs throughout the bioprocessing industry (46, 47), but many manufacturers are sticking to packed-bed chromatography because they do not fully appreciate the advantages of membrane adsorbers in particular applications (48, 49). Whereas packed-bed chromatography utilizes porous adsorbent particles, membrane adsorbers are thin, synthetic microporous or macroporous membranes. They carry functional groups analogous to those on traditional resins, such as quaternary ammonium ions for anion-exchange chromatography (AEX). The membranes are stacked 10–15 layers deep in a comparatively small cartridge, generating a much smaller footprint than columns with a similar output. This reduces buffer consumption but increases the flow rate, because the transport of solutes to their binding sites in a membrane adsorber occurs mainly by convection, while pore diffusion (the predominant mechanism in resins) is minimal (Fig. 14.2). These benefits in terms of buffer consumption and flow rate can reduce process times to 1% or less than those associated with traditional steel columns (50). Overall, the effect is equivalent to shortening a traditional column to a few millimeters in height, allowing large-scale processes to run with only a small pressure drop at very high flow rates. For example, flow-through AEX for mAb polishing with a membrane adsorber can be conducted with a bed height of 4 mm at flow rates of more than 600 cm/h, providing a high frontal surface area-to-bed height ratio (Fig. 14.3). Even though the flow rate is much greater than would be possible with a packed resin, there is sufficient retention time to reduce DNA, most HCP, and many viruses by up to four log reduction values, allowing



**FIGURE 14.2** Mechanistic comparison of solute transport in (a) packed-bed and (b) membrane chromatography. Thick arrows represent bulk convection; thin arrows represent film diffusion, and curly arrows represent pore diffusion.

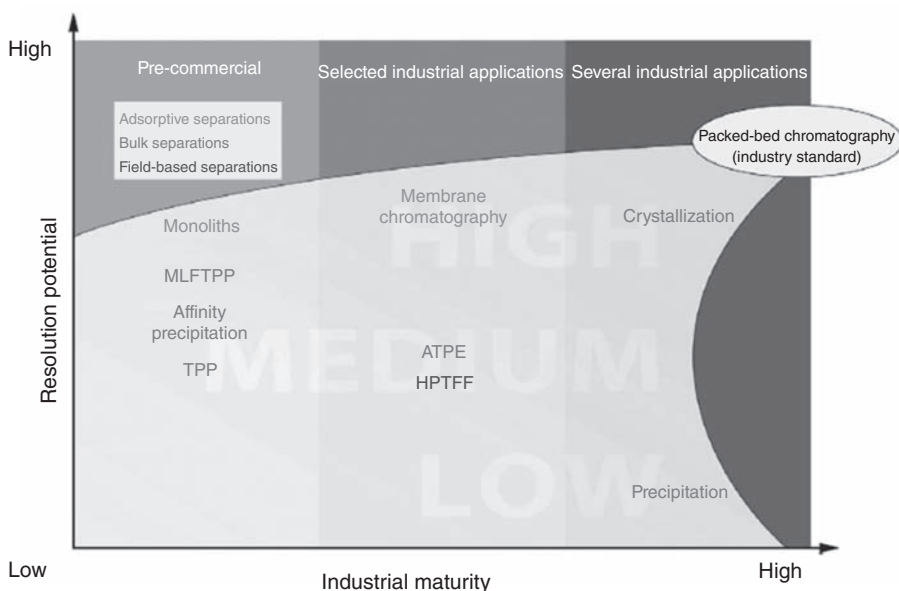


**FIGURE 14.3** Membrane adsorbers have a miniscule bed height compared to columns, which is the functional equivalent of shortening columns to near zero length. There is a correspondingly small pressure drop that allows extremely high flow rates, reducing overall process times up to 100-fold. In this example, both formats have a  $1350\text{-cm}^2$  frontal surface, but the column (a) has a bed height of 15 cm whereas the membrane adsorber (b) has a bed height of just 0.4 cm. The height-to-frontal surface ratio is therefore approximately 100 for the column but is nearer 3500 for the membrane device. Large arrows represent bed height; small arrows show flow of feed stream.

membrane adsorbers to be used not only to separate the product from protein impurities but also as an integrated virus-clearing step (51, 52).

## 14.6 CONCLUSIONS

New technologies such as monoliths, membrane adsorbers, and charged ultra-filtration membranes (see Chapter 16) may provide high-tech solutions to the limited throughput of downstream processing, allowing the replacement of some unit operations currently dominated by packed-bed chromatography (Fig. 14.4). However, it may be that low-tech solutions may also help to lighten the load, with techniques such as precipitation, flocculation, and crystallization reducing the pressure on downstream packed-bed chromatography steps and perhaps helping to reduce the overall number of chromatography steps required. Such approaches are already used with success in the manufacture of conventional pharmaceutical products and industrial proteins (e.g., enzymes for detergents and food processing). Not all these approaches have yet been applied in the manufacture of mAbs, but what works with some biopharmaceuticals may work on others, and the future may see a revision of the chromatography-dominated platform process and its augmentation with



**FIGURE 14.4** A parsing of chromatography alternatives in terms of relative resolution potential and industrial maturity. Packed-bed chromatography is arguably the gold standard. ATPE = aqueous two-phase extraction; HPTFF = high-performance tangential flow filtration; MLFTPP = macroaffinity ligand-facilitated three-phase partitioning; TPP = three-phase partitioning. Reproduced with permission from Elsevier (12).



cheaper, simpler technologies that can be applied on a much larger scale with little impact on the overall cost of goods.

## 14.7 ACKNOWLEDGMENTS

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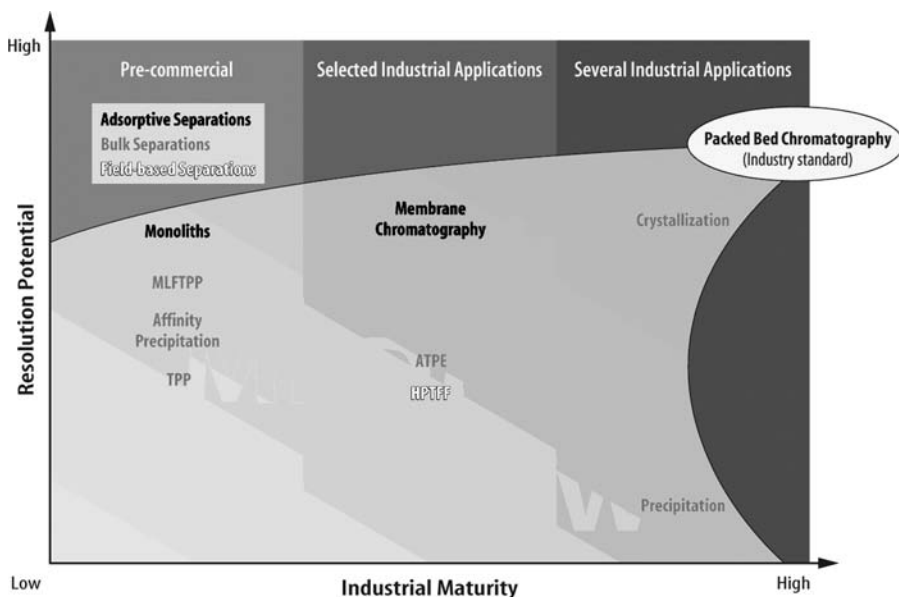
## PROCESS-SCALE PRECIPITATION OF IMPURITIES IN MAMMALIAN CELL CULTURE BROTH

JUDY GLYNN

### 15.1 INTRODUCTION

Many current research initiatives are focused on finding alternatives to column chromatography for the purification of monoclonal antibodies (mAbs). The industry-standard capture resin, Protein A, is expensive, has relatively low capacity compared to ion-exchange (IEX) resins, and does not tolerate cleaning agents, which limits the resin lifecycle. Attempts to develop alternative capture resins have met with some success, but they generally do not offer the same platform potential as Protein A, due to interference by cell culture additives (such as pluronic acid) and the inability to separate product-related impurities such as excess light chain. An initial purification step with the purification capability and generic nature of Protein A is required, but at lower cost and increased capacity. Several nonchromatographic techniques could potentially be developed to replace Protein A as the initial purification step.

In their recent review, Przybycien and colleagues (1) discuss how other industrial applications, such as the purification of polysaccharides, DNA, and viruses, have utilized processes without chromatography for years due to the uniqueness of the target molecule. Could some of these techniques be applied to the purification of mAbs, which also have unique properties compared to the other broth components? The paper classifies the alternative technologies into three distinct areas—bulk, field-based, and adsorptive separation, which

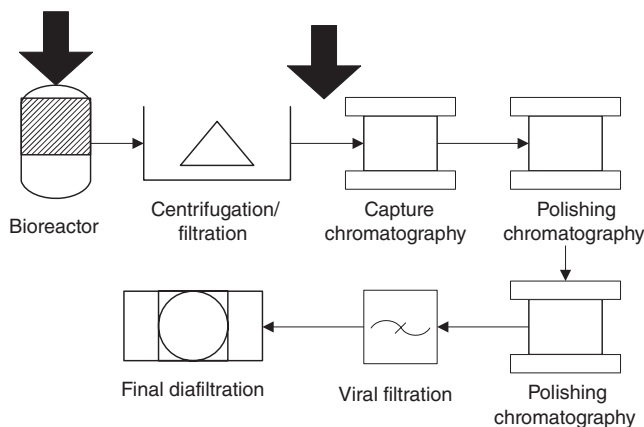


**FIGURE 15.1** A parsing of chromatography alternatives in terms of relative resolution potential and industrial maturity. Reprinted with permission from reference 1. ATPE = aqueous two-phase extraction; HPTFF = high performance tangential flow filtration; MLFTPP = macroaffinity ligand-facilitated three-phase partitioning; TPP = three-phase partitioning.

are categorized according to their industrial maturity vs. resolution potential (Fig. 15.1). Most relevant to this chapter are the bulk separation technologies, which include aqueous two-phase extraction, three-phase partitioning, crystallization, and the technology that this chapter will discuss in detail, precipitation. Precipitation is described as a mature technology with low resolution potential, meaning it is generally nonspecific in terms of its resolving power. However, as discussed above, mAbs have unique properties compared to other components of the broth, which could result in more selective precipitation.

The precipitation of mAbs is discussed in Chapter 14, whereas this chapter focuses on an alternative use of precipitation: the removal of cell culture broth impurities, including DNA and host cell protein (HCP), while leaving the antibody in the supernatant. Where would this precipitation take place in a typical process and how would its potential be realized? What types of precipitation agents would be suitable for use in the manufacture of a parenteral therapeutic agent? These are the questions this chapter will attempt to address.

A standard antibody process often utilized in the industry is shown in Fig. 15.2, which indicates the major potential sites for impurity precipitation. One possibility would be to add the precipitating agent directly to the bioreactor prior to harvest, and to remove the resulting precipitate during the centrifugation or depth filtration step that is often used to remove cells from mammalian culture broth. The other option would be to add the precipitating agent after



**FIGURE 15.2** A standard industry purification process for mAbs indicating potential locations for a precipitation step targeting removal of impurities.

the cells have been removed, and then to remove the resulting precipitate with additional centrifugation or depth filtration. This method has the added value of reducing cellular interaction with the precipitating agent, which could cause the cells to lyse and increase the impurity levels. Both of these options will be discussed later in the chapter. The chapter will also discuss potential precipitating agents in depth, summarize the body of work available for other applications, present the results of recent experiments determining the efficacy of several classes of potential precipitants in impurity removal, and discuss the applications of this technology at process scale.

## 15.2 PRECIPITATION OF DNA AND PROTEIN—OTHER APPLICATIONS

A comprehensive search of the literature reveals that precipitants have been used extensively in other industrial applications, and some of these could potentially be adapted for mAb purification. The precipitants generally fall into two categories—those utilized to purify DNA and proteins, and those used as a precipitating agent to purify antibodies, which could potentially be optimized to remove impurities while leaving the antibody in solution.

Several precipitants described in the literature are used to purify DNA in aqueous solutions. These include, but are not limited to, indium chloride (2), polyaluminum chloride (3), manganese chloride (4), and zinc (5). In addition, several groups have reported on the use of polyamines, such as spermine and spermidine, for DNA precipitation (6, 7). While these techniques are appropriate for precipitation at the laboratory scale as research techniques, they are not suitable for various reasons (e.g., raw material cost, health issues, available chemical grade, ability to detect residual concentrations) for industrial-scale manufacture of pharmaceuticals.

There are, however, precipitants referenced in the literature that could be adapted for use in therapeutic mAb manufacture. One such class of compounds is charged polymers, such as polyethyleneimine (PEI) and polyacrylic acid (PAA), which have proven effective in precipitating both proteins and nucleic acids due to the interaction between the charges on the proteins and the polymers, creating an insoluble complex (8). The use of PEI for the precipitation of nucleic acids has been studied in detail by Cordes and colleagues (9) and by Dissing and Mattiason (10).

In addition to charged polymers, cationic detergents such as cetyltrimethyl ammonium bromide (CTAB) and domiphen bromide (DB) have been used to precipitate cellular DNA (11, 12). Goerke and colleagues (12) were able to demonstrate the use of DB as a DNA precipitant in the purification of adenovirus.

Persson and Lester (13) used 6,9-diamino-2-ethoxyacridine lactate (ethacridine or ethodin) as a precipitant for both DNA and HCP from an *Escherichia coli* homogenate containing antibody fragments. The use of ethacridine (a highly aromatic, cationic dye used as an antiseptic) as a precipitant in antibody purification was first demonstrated by Horejsi and Smetana in 1956 (14) and has been extensively studied since then.

Of particular interest are the numerous descriptions of the use of short-chain fatty acids, such as caprylic acid, for the precipitation of plasma proteins and DNA, as first described by Chanutin and Curnish in 1960 (15). Since then, a large body of work has been completed to determine the optimal parameters for the use of caprylic acid as a precipitant. Several groups have developed conditions for the use of caprylic acid in antibody purification from various starting materials, such as ascites fluid and mammalian serum (16–19). These groups have used caprylic acid to remove the majority of the proteins from the starting material, and then further purified the IgG by methods such as ammonium sulfate precipitation, diethylaminoethyl (DEAE) chromatography, affinity chromatography, or IEX plus high-performance liquid chromatography (HPLC). Parkinnen (20) and Lundblad (21) and their colleagues also showed that caprylic acid could inactivate enveloped viruses. Caprylic acid is also used as a stabilizing agent in albumin solution for intravenous (IV) infusion, so its use with therapeutic agents is already established.

## **15.3 A COMPREHENSIVE EVALUATION OF POTENTIAL PRECIPITANTS FOR IMPURITY REMOVAL**

### **15.3.1 Protocol**

Using this large body of work as a guide, the Mammalian BioProcess Research and Development group at Pfizer Inc. has recently completed an evaluation of several chemical compounds that could potentially be used to precipitate impurities from mammalian cell culture broth containing a mAb. Various cell



**TABLE 15.1 Characteristics of the Chemical Compounds Used in the Pfizer Precipitation Evaluation**

Chemical Name	Synonyms, Abbreviations	Classification
Ammonium sulfate	n/a	Ammonium salt
Caprylic acid	Octanoic acid, caprylate	Short-chain fatty acid
6,9-Diamino-2-ethoxyacridine lactate	Ethacridine, ethodin, rivanol	Aromatic cationic dye
Cetyltrimethyl ammonium bromide	CTAB	Cationic detergent
Domiphen bromide	DB	Cationic detergent
Polyethyleneimine	PEI	Charged polymer
Polyacrylic acid	PAA	Charged polymer
CM dextran	n/a	Charged polymer
DEAE dextran	n/a	Charged polymer
Polymethacrylic acid	PMA	Charged polymer
PEG 4000	n/a	Neutral polymer
1-Butyl-3-methylimidazolium acetate	n/a	Ionic liquid
Tetrabutylammonium bromide	n/a	Ionic liquid
1-Methyl-3-octylimidazolium chloride	n/a	Ionic liquid
1,2,3-Trimethylimidazolium methyl sulfate	n/a	Ionic liquid

n/a = not applicable; CM = carboxymethyl; PEG = polyethylene glycol.

culture broths have been tested, including those produced using either Chinese hamster ovary (CHO) or NS0 cell lines transfected with a construct encoding a fully humanized mAb and grown in an instrumented bioreactor from 1 to 10 L in size. The chemical compounds tested and their classifications are listed in Table 15.1. Some of the listed compounds have never been tested as potential precipitants for mAb purification, while several others have been taken from the literature survey. The new compounds have been evaluated according to their physical properties and an assumption that those characteristics might render them suitable as precipitating agents.

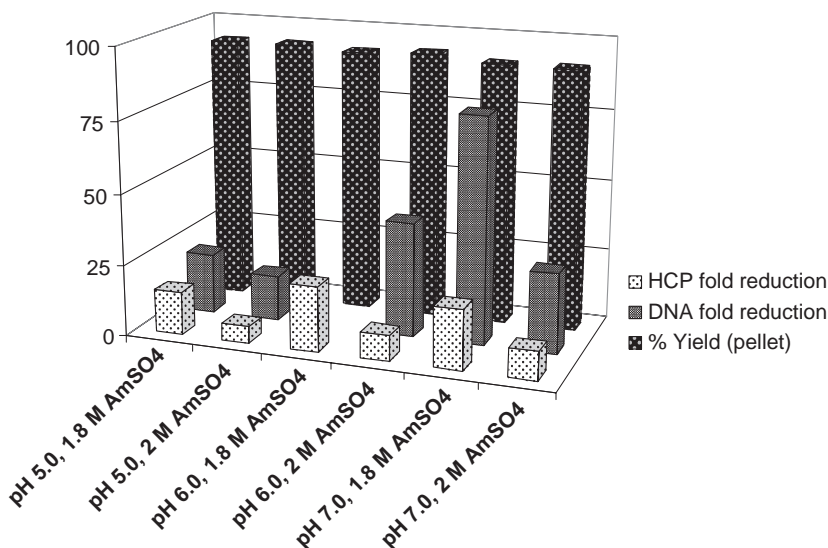
The basic experimental protocol used for the evaluation was as follows. Cells and cellular debris were removed by centrifugation and depth filtration before the study commenced. The broth was adjusted to a final concentration of approximately 1 g antibody/L solution, and the potential precipitant was added to the resulting broth. If required, the pH of the broth was adjusted before the precipitant was added. The broth was incubated for 30–60 min, depending on the formation of a precipitate, then was pelleted in a Sorvall Evolution RC centrifuge for 10 min at  $4000 \times g$ . The supernatant was separated from any precipitate, and if possible, the precipitate was resolubilized. The

resulting fractions of clarified supernatant and resolubilized precipitate were then tested for impurity levels using a standard Cygnus kit (Cygnus Technologies, Southport, NC, USA) for HCP and the threshold assay for DNA. All of the precipitating agents were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA.

### 15.3.2 Ammonium Sulfate Precipitation

Ammonium sulfate precipitation for the purification of mAbs has been extensively documented in the literature [e.g., (22–24)]. This method was performed and used as a standard comparison for antibody purity, regardless of whether the antibody or the impurities were precipitated. In this case, the antibody was precipitated while the impurities remained in solution. After evaluating a range of ammonium sulfate concentrations, 1.8–2.0M was chosen and tested for impurity removal as well as for product yield at various pH values ranging from 4.0 to 7.0. The resulting precipitate was recovered by centrifugation and was washed three times with ammonium sulfate solution to remove additional impurities. The precipitate was then resolubilized and analyzed.

The best results obtained using a variety of NS0- and CHO-produced antibodies are shown graphically in Fig. 15.3. The results indicate a 21-fold reduction in HCP (to 8960 ng HCP/mg antibody) from the starting broth value, and a 79-fold reduction in DNA (to 38,800 pg DNA/mg antibody) with a yield of



**FIGURE 15.3** Best results obtained from ammonium sulfate precipitation using an NS0-produced antibody. No DNA data are available for pH 6.0, 1.8M ammonium sulfate conditions.

91%. The fold reduction in impurities was calculated by dividing the impurity value in the broth by the impurity value in the antibody phase (supernatant or resolubilized precipitate, as appropriate).

### 15.3.3 Polymer Precipitation

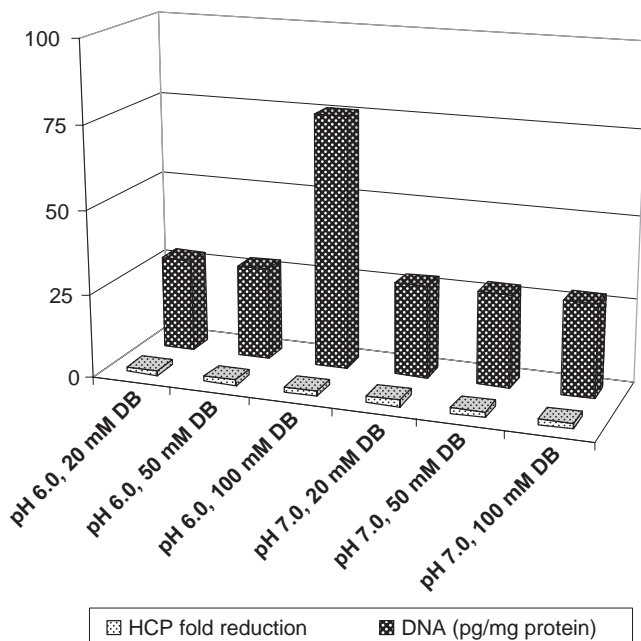
All of the charged polymers listed in Table 15.1 were screened as precipitating agents at concentrations of 0.01%–5% (v/v) using both CHO- and NS0-produced mAbs. Only PEI and PAA resulted in visible precipitation, and only at the higher concentrations. No loss of antibody was detected in the supernatant for either polymer, but the PAA supernatant could not be filtered using standard means, and the PAA itself interfered with the HCP and DNA assays. Additional experiments were performed using PEI with and without the addition of the neutral polymer PEG 4000. The neutral polymer was added potentially to enhance precipitation by reducing the hydration level, which could theoretically cause the protein-charged polymer complex to precipitate. However, none of the conditions reduced HCP levels significantly (maximum 2.5-fold reduction) as compared to the ammonium sulfate standard. DNA reduction was not tested due to the marginal results from the HCP analysis and the significant filtration issues noted above.

### 15.3.4 Precipitation with Ionic Liquids

Ionic liquids are organic salts (or mixtures containing an organic component) that remain in liquid form at low temperatures. Many are water soluble, which makes them suitable for use in manufacturing processes, and their organic components are thought to confer hydrophobic properties that could enhance their efficiency as precipitating agents. Several ionic liquids of different classes were screened, but only four were actually tested as precipitants because of solubility and filtration issues (see Table 15.1). They were evaluated using both CHO and NS0 cell culture broths, at various pH and concentration values. None of the tested conditions reduced HCP values significantly compared to the ammonium sulfate standard (the best result obtained was a 1.7-fold reduction), and therefore DNA testing was not performed. Given their high cost, limited availability, and solubility issues at higher concentrations, further testing was abandoned.

### 15.3.5 Precipitation with Cationic Detergents

Both CTAB and DB were tested extensively, initially using only the NS0-derived antibody. Although neither compound reduced the amount of HCP, both were effective in the precipitation of DNA. The best results obtained with CTAB (5 mM) were a 164,000-fold reduction in DNA levels, down to ~190 pg DNA/mg antibody, which is significantly better than that obtained with the ammonium sulfate standard.

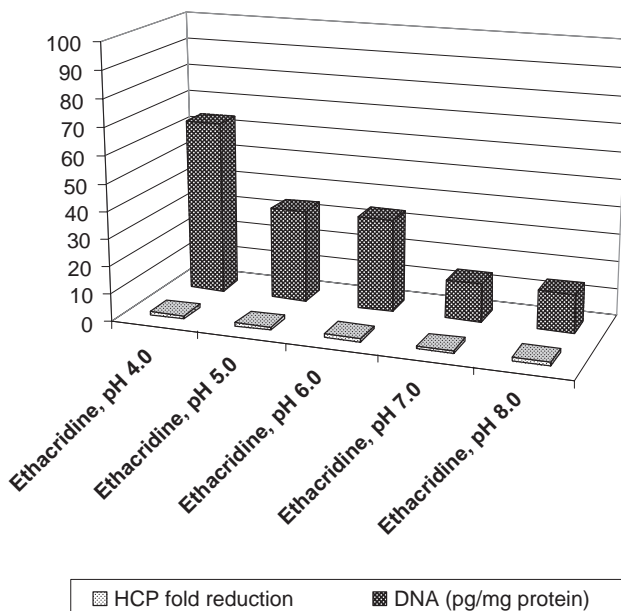


**FIGURE 15.4** Best results obtained from DB precipitation of impurities in CHO-produced mAb cell culture broth.

DB (5 mM) performed even better than CTAB, reducing DNA to undetectable levels at all pH values tested, with very high recovery of mAb in the supernatant (>95%). Additional experiments were performed on a CHO-produced antibody, and the results are summarized in Fig. 15.4. There was no significant reduction in HCP levels, but DNA levels were below the level of detection for the assay based on the lowest dilution necessary to obtain a spike recovery that passed the assay specification (note that HCP levels are shown as a fold reduction, whereas DNA levels are actual values). For all of the DB concentrations and pH values tested, the starting value was  $>11 \times 10^6$  pg DNA/mg antibody, showing that DB is very effective at precipitating DNA while leaving the antibody product in solution.

### 15.3.6 Ethacridine Precipitation

The aromatic, cationic dye 6,9-diamino-2-ethoxyacridine lactate (ethacridine) was tested at several pH values and at concentrations of 0.1%–2.0% (w/v). The dye was added to both NS0 and CHO cell broths containing a mAb. Although all of the ethacridine concentrations and pH values resulted in some degree of precipitation, only the highest concentrations at each pH value were analyzed for impurity reduction. These results are summarized in Fig. 15.5. Again, as for DB, there was no significant reduction in HCP values (presented

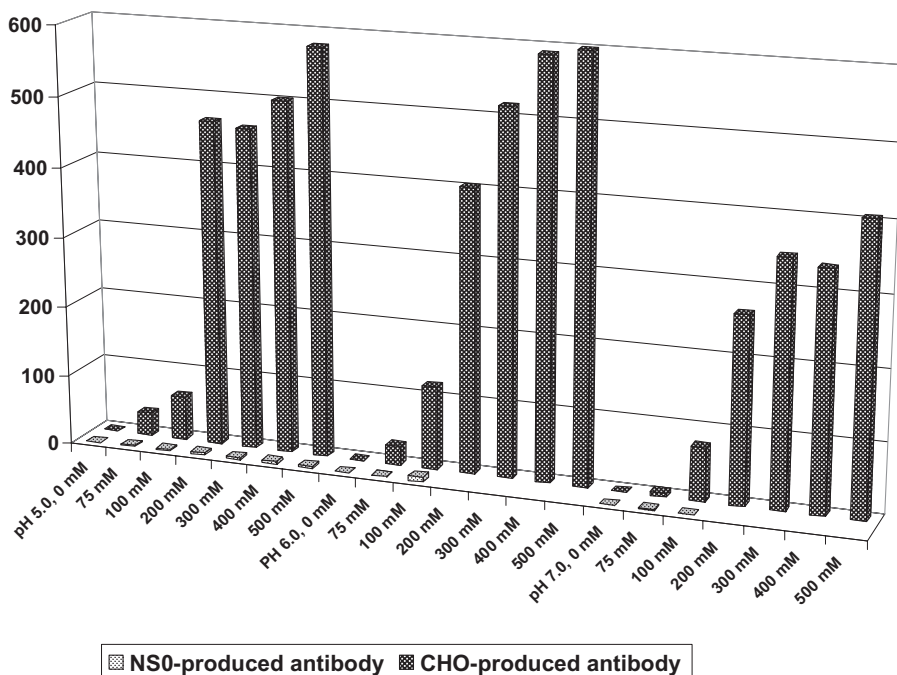


**FIGURE 15.5** Best results obtained from ethacridine precipitation of impurities in CHO-produced mAb cell culture broth.

as fold reduction), but the DNA reduction was significant. Excepting the value obtained at pH 4.0, all of the DNA values were essentially below the level of detection, as compared to a starting broth value of over  $31 \times 10^6$  pg DNA/mg antibody. However, the intense yellow coloration of ethacridine interfered with the Protein A HPLC assay and the yield could not be determined. Even after centrifugation and filtration, the supernatant continued to precipitate out over several days. This process could be optimized by varying the ethacridine concentration or temperature in order to complete the precipitation in a reasonable time frame, but further evaluation would be necessary to determine whether the remaining ethacridine in the supernatant can be removed in the purification process downstream of the precipitation, and what effect it will have on the subsequent chromatography steps. It is possible that the ethacridine could be removed using an ultrafiltration step prior to chromatography.

### 15.3.7 Caprylic Acid Precipitation

Initial experiments were performed using an NS0-produced antibody at low pH values (3.4–4.8) and a caprylic acid concentration of 30mM, based on information from the literature. Although there was no reduction in HCP levels, DNA levels were undetectable at the higher pH values tested. Additional testing for both NS0- and CHO-produced antibodies at pH 4.0–6.0 and caprylic acid concentrations of up to 100mM confirmed that, for the



**FIGURE 15.6** HCP fold reduction values for caprylic acid precipitation utilizing both a CHO- and NS0-produced antibody.

NS0-produced antibody, DNA was significantly reduced at the lower pH values while HCP was not affected. However, for the CHO-produced antibody, both HCP and DNA were significantly reduced at all pH values tested.

Additional experiments confirmed that, although DNA values were reduced below the level of detection for both NS0- and CHO-produced antibodies, the impact of caprylic acid on HCP levels differed significantly according to the cell line (Fig. 15.6). For the CHO-produced antibody, the reduction in HCP levels continued to increase in line with the caprylic acid concentration. However, for the NS0-produced antibody, there were no further significant improvements in HCP clearance at caprylic acid concentrations above 100 mM. The yield for the CHO-produced samples averaged 92%, and caprylic acid precipitation performed at pH 6.0 with 500 mM caprylic acid (higher concentrations produced solubility issues) resulted in the best overall performance (650-fold reduction in HCP to 160 ng/mg antibody, and 6475-fold reduction in DNA to 700 pg/mg antibody). These results were significantly better than those obtained using ammonium sulfate precipitation.

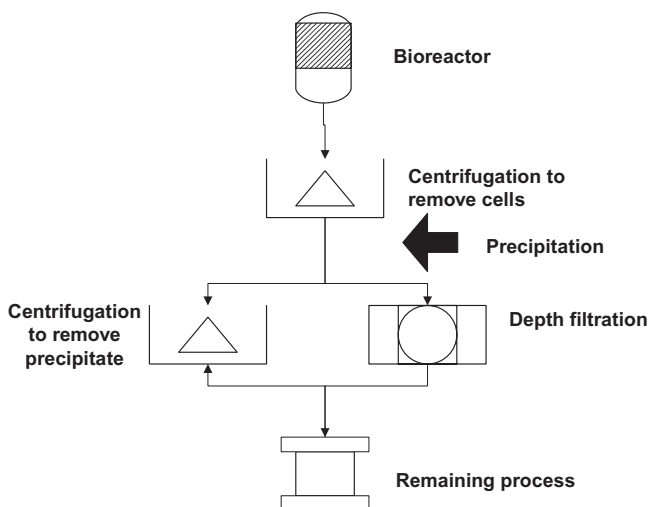
A few observations made during these experiments make for interesting engineering challenges. At the higher concentrations of caprylic acid (generally 300 mM and above), the resulting precipitation actually produces a three-phase system, with a pellet forming at the bottom of the tube, a supernatant

in the middle, and a fluffy precipitate at the top. This top layer can be removed by filtration once the supernatant is decanted, but it is unclear how it would affect an attempt to remove the pellet by centrifugation. Additionally, with the formation of the fluffy layer, filtration of the supernatant was very difficult, and would require the development of a filtration train to remove it (the experiments discussed above involved a standard two-layer sterilizing-grade filter, such as the Sartorius Sartopore 2, which is a  $0.45\text{ }\mu\text{m}$  over  $0.2\text{ }\mu\text{m}$  filter). Utilizing a lower caprylic acid concentration to negate the three-phase and filtration effects while still retaining the ability to remove impurities could be an appropriate process compromise.

## 15.4 INDUSTRIAL-SCALE PRECIPITATION

Given the results discussed above, how could such a process be scaled? What technologies could be employed, and where might it fit in a standard mAb purification scheme? If the goal is to remove the majority of the impurities before the initial chromatography step, which might have the added advantage of increasing the capacity and/or lifetime of the resin, then it must be located at the harvest step, where two major technologies could be utilized, centrifugation or filtration (Fig. 15.7). The precipitation could also be carried out in the bioreactor, but the effect of the precipitating agent on the cells would have to be determined.

The type of centrifugation most commonly used in mAb processes is disk-stack centrifugation, which is ideal for separation when the solids



**FIGURE 15.7** Potential purification schemes utilizing a precipitation step.

concentration is low, or where smaller particles are present (25). This technology and its adaptation to mammalian cell culture broth has been described extensively in the literature and has been presented at conferences (26–29). In order to develop a centrifugation step to remove the precipitate, a scale-down model would need to be developed (30,31). Once this model is available, the parameters affecting separation could be studied and optimized. For centrifugation, such parameters would include the sigma factor, the relative densities of the materials to be separated, angular velocity, viscosity, feed flow rate, and temperature (32). In addition, solids removal from the bowl during continuous-flow centrifugation could also play an important role in centrate quality (33).

The other option is depth filtration, which is also used extensively to process mAbs and related molecules (34, 35). Depth filtration has advantages over centrifugation, such as the removal of DNA (36) and HCP (37) under certain circumstances. However, it is no trivial matter to screen the wide array of depth filters currently available and to determine the correct one for the application, and then to determine the optimal parameters for processing, which include the pH and ionic strength of the feed fluid, feed flux, membrane area, and operating pressure (38). New technologies targeting better fluid dynamics and ease of operation may increase the use of depth filtration.

If a technique can be adapted for precipitate removal following treatment with an agent such as caprylic acid, the technology could be very useful. Early removal of significant amounts of HCP and DNA could result in the adaptation of a shorter purification process, such as hydrophobic interaction chromatography (HIC) followed by Q membrane chromatography (see Chapter 14), or better performance and longer life of the capture resin.

## 15.5 COST OF GOODS COMPARISON

If precipitation could potentially replace Protein A as the initial purification step, how would it compare in terms of cost? Assuming a process starting with a 20,000-L reactor with a titer of 2 g/L, a Protein A resin capacity of 30 g/L, and three cycles per purification run, the Protein A column would be 1.8 m in diameter with a bed height of 18 cm and a bed volume of 460 L. The cost of goods determination per lot for Protein A vs. precipitation with caprylic acid is outlined in Table 15.2. The comparison shows that at the concentrations of caprylic acid evaluated, Protein A resin is more cost-effective due to its longer life cycle (estimated at 100 runs). One option to reduce the cost of goods for caprylic acid precipitation would be to concentrate the broth after cell removal, or to determine the actual concentration of caprylic acid needed per gram of antibody as opposed to volume of broth. If a lower concentration of caprylic acid could be used, it would also produce a two-phase separation instead of the three-phase separation described above, which would confer processing simplicity as well. In addition, the precipitation step would not require packing,



**TABLE 15.2 Cost of Goods Comparison of Protein A Chromatography vs. Caprylic Acid Precipitation**

Raw Material	Lifetime	Cost/L, \$	Total Volume Needed, L	Approximate Cost per Lot, \$
rProtein A Sepharose FF Resin (GE Healthcare)	100 cycles	7770	460 per lifecycle	108,000
Caprylic acid, 500mM	n/a	250	1440 per lot	360,000
Caprylic acid, 200mM	n/a	250	577 per lot	144,000

qualifying, or cleaning of the column, and no life cycle validation would be required.

## 15.6 SUMMARY

Alternatives to standard chromatography for the purification of mAbs have been discussed in detail in the literature. One such alternative, precipitation, is used extensively in other applications for the purification of polysaccharides, DNA, and viruses, and may be adaptable to antibody purification. The technology could be manipulated to precipitate out the antibody, as is the case with ammonium sulfate, or to reduce impurity levels while leaving the antibody in solution.

Several potential precipitating agents that have been used in other applications were evaluated for their ability to purify antibodies by reducing HCP and DNA. Two cationic detergents, CTAB and DB, were able to precipitate DNA much more effectively than ammonium sulfate. In addition, the short-chain fatty acid caprylic acid was able to remove both DNA and HCP very efficiently for CHO-produced antibodies, but less efficiently for NS0-produced antibodies. A precipitation step targeting the removal of impurities could be developed using centrifugation and/or depth filtration technology during the harvest operation, but would need to be optimized to ensure the cost of goods compared favorably with a standard chromatographic technique such as Protein A. If these engineering challenges can be addressed, precipitation may prove to be a valuable tool for antibody purification, although the long-term effects of a precipitating agent on antibody stability would need to be investigated.

## 15.7 ACKNOWLEDGMENTS

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## **CHARGED ULTRAFILTRATION AND MICROFILTRATION MEMBRANES IN ANTIBODY PURIFICATION**

MARK R. ETZEL

### **16.1 INTRODUCTION**

Ultrafiltration and microfiltration membranes have been used since the dawn of the biotechnology industry and have been integrated into every process for the manufacture of monoclonal antibodies (mAbs). The process-scale purification of mAb products is highly profitable, and companies have invested large amounts of capital in equipment for membrane separations and in knowledgeable personnel with the experience needed to design, operate, and troubleshoot membrane separation processes. It is probably safe to say that membrane separations will always play a big part in the manufacture of mAb products.

There have been new developments in membrane separation processes every year since the technology was first introduced, including new ultrafiltration membranes with sharper molecular weight cutoffs, higher flux, less fouling, and greater uniformity. Microfiltration membranes have started to compete with centrifugation for clarification and cell removal steps. A fundamental understanding of the principles of ultrafiltration and microfiltration membrane separation is an active area of research, bearing much fruit. We now understand the principles far better than before, and that knowledge has led to new membrane products, new process-scale equipment, and new strategies for operating that equipment.

Adding a charge to existing membrane products to create charged ultrafiltration and microfiltration membranes would seem like an obvious way to expand the potential of this huge investment in technology and in people. However, this change has been a slow endeavor. Charged microfiltration membrane products are also known as membrane adsorbers (and the separative process is known as membrane chromatography). Although first introduced in the late 1980s, these products were slow to be accepted by the bioprocessing industry, and languished for a marketplace. There has been renewed interest in membrane adsorbers recently, particularly because of their ability to remove impurities such as viruses, nucleic acids, endotoxins, and host cell protein (HCP) from mAb products, and this may expand the use of membrane adsorbers for other applications. Charged ultrafiltration membranes are still a novelty and curiosity, and then only among researchers in the process-scale purification of mAb products.

An analogy can be drawn between chromatography beads and ultrafiltration/microfiltration membranes. Chromatography beads have been used first in the biotechnology industry for gel filtration, and have been purposely neutral in charge. Separation has been based on the sieving effect or on a size-based separation. Later, a charge has been added to the beads to form ion exchangers, and ion-exchange (IEX) chromatography has become a mainstay of process-scale mAb manufacture (see Chapters 5 and 7). Will ultrafiltration and microfiltration membranes follow a similar route?

In this chapter, charged ultrafiltration and microfiltration membranes are considered together for the reasons expressed above. Although each technology is discussed separately, the shared feature is that one simply adds a charge to existing ultrafiltration and microfiltration membrane products.

## 16.2 CHARGED ULTRAFILTRATION MEMBRANES

The separation of proteins by charged ultrafiltration membranes was first reported in a landmark publication (1), which showed that by adding either a positive or a negative charge to a standard ultrafiltration membrane, it was possible to separate myoglobin and cytochrome C by setting the buffer pH near the isoelectric point (pI) of one of the proteins, allowing it to permeate the membrane, and thereby rejecting by electrostatic repulsion the protein that carried a like charge to the membrane. Thus, they demonstrated that it was possible to separate proteins with different pIs using a charged ultrafiltration membrane, even though the sizes of the proteins were nearly the same.

Zydney and van Reis have done much to better understand the theory and application of charged ultrafiltration membranes for protein separation (2, 3). The following sections recount and highlight pertinent aspects of that work, and include a case study of wholly new data obtained in our laboratory to illustrate the technology and basic principles.

### 16.3 CONCENTRATION POLARIZATION AND PERMEATE FLUX

The flux of clean water through an ultrafiltration membrane is given by the membrane hydraulic permeability coefficient ( $L_p$ ):

$$L_p = J_v / \Delta P \quad (16.1)$$

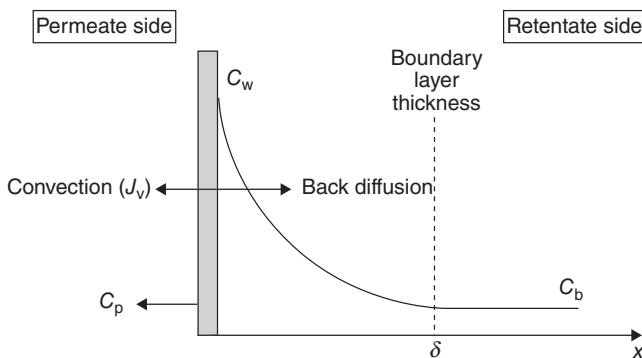
where  $J_v$  is the permeate flux, and  $\Delta P$  is the transmembrane pressure drop (2). For uniform cylindrical pores, the following expression applies:

$$L_p = \frac{\epsilon r^2}{8\mu\delta_m}, \quad (16.2)$$

where  $\epsilon$  is the membrane void fraction,  $\delta_m$  is the membrane thickness,  $\mu$  is the solvent viscosity, and  $r$  is the pore radius. The hydraulic permeability coefficient increases with decreasing viscosity or increasing temperature. For example, the viscosity of water decreases almost threefold from 1.567 to 0.547 cP as temperature increases from 4 to 50 °C.

It is important to measure and record the clean water flux of each new membrane unit before first use, and then to repeat the measurement periodically, after reuse and cleaning. If the cleaning procedure is effective, then the clean water flux should return to 75%–90% of the initial value. When the clean water flux after cleaning drops to less than 50% of the initial value, then the membrane is typically replaced.

When a feed solution contains protein, the flux is less than the clean water flux because of the effect known as concentration polarization (Fig. 16.1). As pressure drives solvent flow through the membrane, convection transports solutes to the upstream surface of the membrane, causing solutes that do not pass through the membrane to build up. The solute concentration in solution is highest at the wall of the membrane ( $C_w$ ) and falls to the bulk solution value ( $C_b$ ) over the thickness of the concentration boundary layer ( $\delta$ ).



**FIGURE 16.1** Schematic diagram of protein transport by convection and diffusion inside of the concentration polarization boundary layer of thickness  $\delta$ .

### 16.4 STAGNANT FILM MODEL (SFM)

Concentration polarization has been explained using the SFM, first proposed by Michaels (4), and is based on the following mass balance:

$$J_v C - J_v C_p = -D \frac{dC}{dx} \quad (16.3)$$

where  $C$  is the solute concentration upstream at a distance  $x$ ;  $C_p$  is the solute concentration in the permeate stream, and  $D$  is the solute diffusion coefficient. The first term on the left of the equation accounts for solute moving toward the membrane by convection; the second term on the left accounts for solute leaking through the membrane by convection, and the term on the right accounts for solute moving away from the membrane by diffusion according to Fick's law. At steady state, the solute transport rate by back diffusion is exactly balanced by the net rate of forward solute transport to the membrane. When Equation 3 is integrated over the boundary layer thickness (i.e., from  $C = C_w$  at  $x = 0$  to  $C = C_b$  at  $x = \delta$ ), then

$$J_v = k \ln \frac{(C_w - C_p)}{(C_b - C_p)}, \quad (16.4)$$

where the solute mass transfer coefficient ( $k$ ) is equal to the ratio of the solute diffusion coefficient to the boundary layer thickness ( $k = D/\delta$ ).

Zydney (5) provides an excellent explanation for the lack of rigor in the above derivation. Nevertheless, for over 40 years, the SFM has been used almost universally to describe bulk mass transfer and concentration polarization in ultrafiltration systems. Zydney provides a more rigorous mathematical derivation of the SFM (5). Despite its limitations, the SFM works well regardless of how it is derived.

### 16.5 OSMOTIC PRESSURE MODEL

The osmotic pressure model is an alternative to the SFM that also explains the flux decline that occurs when using protein solutions vs. clean water (2, 6). In this model, the buildup of protein at the wall of the membrane ( $C_w$ ) creates an osmotic pressure ( $\Delta\pi$ ) that counteracts the applied pressure ( $\Delta P$ ):

$$J_v = L_{fm} (\Delta P - \sigma \Delta \pi), \quad (16.5)$$

where  $L_{fm}$  is the hydraulic permeability of the membrane and any adsorbed or deposited material on it (fm = fouled membrane), and  $\sigma$  is the osmotic reflection coefficient, a measure of membrane selectivity. For a fully retentive membrane  $\sigma = 1$ . For  $\Delta\pi$ , a simple polynomial expression can be used where  $\alpha$  and  $\beta$  are constant coefficients:



$$\Delta\pi = \alpha C_w + \beta C_w^2. \quad (16.6)$$

Thus, the solvent flux declines as protein builds up on the front surface of the membrane ( $C_w$ ). Eventually, a pressure-independent region develops where the flux does not increase further with further increases in pressure, because  $C_w$  rises to the point where  $\Delta\pi$  is comparable to  $\Delta P$  in Equation 5 and the driving force approaches zero. In order to maintain the flux as close as possible to the clean water flux, one must keep  $C_w$  low, as discussed later.

## 16.6 MASS TRANSFER COEFFICIENT

The mass transfer coefficient used in Equation 4 can be calculated for a stirred-cell ultrafiltration system using the following correlation (7, 8):

$$\left(\frac{kr_c}{D}\right) = 0.27 \text{Re}^{0.567} Sc^{0.33}, \quad (16.7)$$

where  $\text{Re} = \Omega r_c^2 / \nu$  is the Reynold's number;  $Sc = \nu / D$  is the Schmidt number;  $r_c$  is the radius of the stirred cell;  $\Omega$  is the stirring speed in radians;  $D$  is the diffusion coefficient of the protein in the bulk solution, and  $\nu$  is the kinematic viscosity.

The mass transfer coefficient in a cross-flow membrane module for fully developed laminar flow is a function of the length of the module ( $L$ ), the diffusion coefficient of the protein ( $D$ ), and the shear rate ( $\gamma$ ) as given by Ghosh and Cui (9):

$$k = 0.816 \left( \frac{\gamma D^2}{L} \right)^{0.33}, \quad (16.8)$$

where the shear rate is given by  $\gamma = 6\nu/h$  for flow of velocity  $\nu$  through a rectangular channel of height  $h$ . The mass transfer coefficient increases with increasing cross-flow velocity and diffusion coefficient, and decreasing module length and channel height. In other words, to increase the mass transfer coefficient, use a high retentate flow rate, small protein, high temperature, thin channel, and short channel. For scale-up, all these parameters should remain the same.

## 16.7 SIEVING COEFFICIENT

The sieving coefficient is a fundamental measure of ultrafiltration membrane performance. Two definitions are frequently used, the intrinsic sieving coefficient ( $S_i$ ),

$$S_i = C_p / C_w, \quad (16.9)$$

and the observed sieving coefficient ( $S_o$ ):

$$S_o = C_p / C_b. \quad (16.10)$$

These two definitions can be substituted into Equation 4 to eliminate the concentration variables:

$$J_v = k \ln \frac{(C_p/S_i - C_p)}{(C_p/S_o - C_p)} = k \ln \frac{(1/S_i - 1)}{(1/S_o - 1)}, \quad (16.11)$$

which can be rearranged to the form

$$S_o = \frac{S_i}{S_i + (1 - S_i) \exp(-J_v/k)}. \quad (16.12)$$

Another useful form of Equation 4 is explicit for the wall concentration at the surface of the membrane:

$$C_w = C_p + (C_b - C_p) \exp(J_v/k). \quad (16.13)$$

The wall concentration of protein increases with decreasing  $k$ , and increasing  $C_b$  and  $J_v$ .

The ratio of sieving coefficients is called the selectivity:

$$\Psi = S_{o,1} / S_{o,2}, \quad (16.14)$$

where  $S_{o,1}$  and  $S_{o,2}$  are the observed sieving coefficients of the less and more retained proteins, respectively. In general, protein fractionation is enhanced when the sieving coefficient of one protein is much greater than the other. The selectivity is governed by the ratio of the flux to the mass transfer coefficient because of Equation 12. Therefore, there may be an optimum in the selectivity vs. flux as shown theoretically by Ghosh and Cui (9).

## 16.8 DIFFUSION-CONVECTION MODEL

The sieving coefficient can be calculated using the convection-diffusion mass balance across the membrane (10):

$$S_i = \frac{S_\infty e^{Pe}}{S_\infty + e^{Pe} - 1}, \quad (16.15)$$

where  $S_\infty$  is the value of the intrinsic sieving coefficient found at infinite Peclet number (11):

$$Pe = \frac{S_\infty J_v \delta_m}{D_m}, \quad (16.16)$$

where  $\delta_m$  is the membrane thickness, and  $D_m$  is the effective diffusion coefficient of protein through the membrane. As the flux ( $J_v$ ) increases, the intrinsic sieving coefficient  $S_i$  approaches the value of the asymptotic sieving coefficient  $S_\infty$ .

There are serious limitations to the SFM and diffusion–convection models. First, neither model accounts for the effect of more than one protein in solution. For example, if two proteins are present, then the protein with the smallest sieving coefficient will build up on the surface of the membrane and the other protein must pass through this layer in addition to passing through the membrane. The protein layer formed by the protein with the smallest sieving coefficient may act as a finer filter than the membrane itself, that is, for the protein having the largest sieving coefficient. This effect is not accounted for in the models. Second, the diffusion–convection model does not account for the effects of concentration polarization. Thus, neither model is strictly valid and may not provide reliable and accurate predictions.

## 16.9 SCALE-UP STRATEGIES AND THE CONSTANT WALL CONCENTRATION ( $C_w$ ) APPROACH

Scale-up has been accomplished by many different methods, only a couple of which utilize basic principles. Most scale-up involves keeping either the trans-membrane pressure ( $\Delta P$ ) or less commonly, the flux ( $J_v$ ) constant, and then increasing the module length to increase scale. Two newer approaches keep either  $C_w$  constant or the channel length, shear rate, and flux constant (linear scale strategy).

Frequently, the  $\Delta P$  is selected to maximize flux by operating in the pressure-independent region of the flux vs.  $\Delta P$  curve. In this situation, the effect of concentration polarization is significant, and the concentration of proteins at the membrane wall can soar into the region of protein insolubility, aggregation, and precipitation. Furthermore, inevitable variations in membrane permeability during manufacture result in a different flux at a given  $\Delta P$  for each individual membrane. Therefore, scaling up based on constant  $\Delta P$  will result in different values of the flux and consequently in the concentration of proteins at the membrane wall. On the other hand, scale-up based on constant flux ignores the effect of the mass transfer coefficient on the wall concentration. Increasing the channel length to increase membrane area or increasing the channel flow velocity to increase throughput will both change the wall

concentration. Scale-up based on constant wall concentration (6) is a more intelligent, fundamental, and rigorous approach.

The constant  $C_w$  approach uses the SFM or the osmotic pressure model to control the flux. During a protein concentration operation using a fully retentive membrane ( $S_o \approx 0$ ), the protein concentration in the bulk tank increases from the initial value of  $C_0$  at batch volume  $V_0$  to a final value of  $C_b = C_0 V_0 / V$  when the final concentrate volume decreases to  $V$ . The volume concentration ratio ( $VCR = V_0 / V$ ) increases during a concentration operation, and it is used to calculate the flux vs. time relationship that maintains  $C_w$  constant using the SFM relation:

$$J_v = k \cdot \ln \left( \frac{C_w / C_0}{VCR} \right). \quad (16.17)$$

The flux at any two times, while holding  $C_w$  constant, does not depend on the value of  $C_w$ :

$$J_{v,2} = J_{v,1} - k \cdot \ln(VCR_2 / VCR_1). \quad (16.18)$$

Therefore, a semilog plot of flux vs. the volume concentration ratio is a straight line with a negative slope equal to the mass transfer coefficient.

One could argue that the constant  $C_w$  approach solves a problem that does not exist in antibody manufacture, because most mAbs are very soluble and are unlikely to aggregate at high  $C_w$  values. Furthermore, existing process-control skids cannot control permeate flux;  $\Delta P$  is controlled instead. It would be difficult to reduce the permeate flux over time without a pump and flow rate detector on the permeate stream. In addition, because the membranes selected for concentration operations are very retentive ( $S_i \approx 0$ ), essentially no antibody leakage occurs even at high  $C_w$  values.

There are some flaws in this argument. First, the constant  $C_w$  approach may allow the use of a less-retentive membrane. For example, a single membrane type often has a twofold lot-to-lot difference in permeability (6). Using constant  $\Delta P$  control, flux would also differ twofold, increasing  $C_w$  as well as losses for the more permeable membrane lot. More retentive membranes would be required to make certain losses were acceptable in the case a more permeable membrane lot was installed on a given day. However, constant  $C_w$  control would compensate for lot-to-lot variations in permeability, allowing the use of a less-retentive membrane. Second, protein fractionation requires that one protein permeates the membrane more than another. Allowing  $C_w$  to increase to large values will form a protein layer on the membrane surface that may act as a finer filter than the membrane itself, preventing fractionation. This is especially important when diafiltration is used to wash impurities out of a mAb product. Third, fundamental principles support the  $C_w$  approach to scale-up. A systematic theoretical approach to scale-up is likely to be more reliable than an Edisonian approach.

## 16.10 PROTEIN FRACTIONATION USING CHARGED ULTRAFILTRATION MEMBRANES

When the membrane surface contains a net charge, then the sieving coefficient of proteins that also have a net charge is altered according to whether the charges are alike or different (12, 13). Protein fractionation is enhanced by using a highly charged membrane, low ionic strength solutions, and a pH that causes one protein to be highly charged compared to another protein. Broadly speaking, the sieving coefficient is greatest when the buffer pH is near the pI of the protein, and when the salt concentration is elevated sufficiently to shield the charges on the protein and membrane. When the protein and membrane charges are alike, then electrostatic repulsion reduces the sieving coefficient to a value lower than would be the case in the absence of electrostatic interactions. When the charges are different, then the charged proteins adsorb to the oppositely charged membrane and form a dynamic membrane that has the same charge as the protein molecules in solution. This also causes electrostatic repulsion and a decrease in the sieving coefficient.

Protein fractionation by ultrafiltration has become a promising new unit operation since the development of charged ultrafiltration membranes (1, 3). In the past, ultrafiltration was a separation process based solely on differences in molecular size. Charged ultrafiltration membranes expanded that basis of separation to both molecular size and charge by altering the charge on the membrane in addition to the pore size, and by altering the charge on the proteins by adjusting the buffer pH. As a rule of thumb, a positively charged protein is rejected most by a positively charged membrane. Because therapeutic mAbs have a pI of 8–10, rejection at neutral pH is best accomplished using a membrane carrying a positive charge. A straightforward method for placing a positive charge on an ultrafiltration membrane was described by van Reis (3) wherein regenerated cellulose membranes were reacted with (3-bromopropyl)trimethylammonium bromide in 100 mM NaOH at room temperature overnight. Such membranes can be modified while still in the membrane holder using a recirculation flow method. Negatively charged membranes were made using a solution of 3-bromopropanesulfonic acid in 100 mM NaOH.

Using a mAb of pI = 8.9, van Reis (3) showed that the sieving coefficient for uncharged and positively charged ultrafiltration membranes with a 1000-kD molecular weight cutoff dropped from  $S_o = 0.9$  to 0.006, respectively, at pH 4.5. In other words, an over 100-fold reduction in sieving coefficient occurred when charge was added to the ultrafiltration membrane.

## 16.11 CASE STUDY

The advantages of charged ultrafiltration membranes are illustrated below using the following system. Two whey proteins,  $\beta$ -lactoglobulin (BLG) and

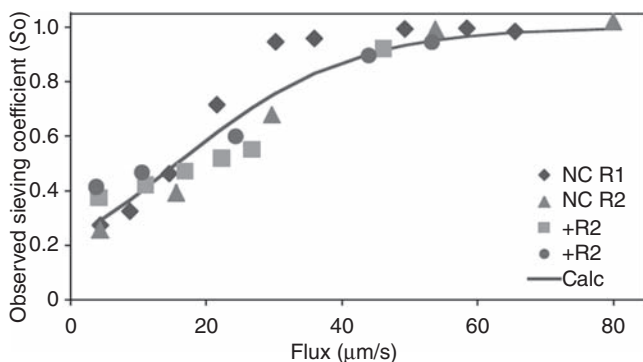
glycomacropeptide (GMP), were separated individually and in binary mixtures using both charged and uncharged regenerated cellulose ultrafiltration membranes.

### 16.11.1 Methods

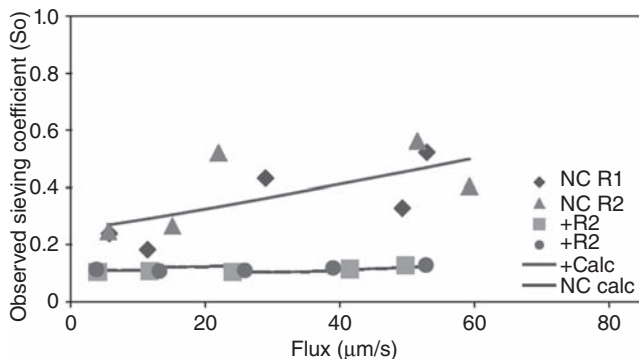
BLG (catalog number 100363, MP Biomedicals, Solon, OH, USA) is an 18.6-kDa protein of  $pI = 5.1$  that forms dimers at pH 5.5–7.5, tetramers at pH 3.5–5.2, and monomers at pH < 3.0. GMP (BioPURE-GMP, Davisco Food Intl., Eden Prairie, MN, USA) is an 8.6-kDa protein of  $pI < 3.6$  that is a monomer regardless of the buffer pH. These proteins were separated using 30-kDa regenerated cellulose membranes (YM30, Millipore, Billerica, MA, USA). One membrane was uncharged, and the other membrane was given a positive charge by reacting it with (3-bromopropyl)trimethylammonium bromide using the method of van Reis (3). Membranes were placed into a stirred ultrafiltration cell (model 8010, Millipore) operated at 600 rpm. Protein solutions of approximately 1 g/L were prepared in 25 mM sodium phosphate, pH 3.0, with NaCl added to achieve a conductivity of 4.0 mS/cm. Solutions were microfiltered [0.22- $\mu$ m polyvinylidene fluoride (PVDF) syringe filter, Millipore] prior to use. Protein concentrations were measured using a spectrophotometer at 214 and 280 nm. GMP does not absorb at 280 nm because it contains no aromatic amino acids. This unique feature was used to determine the concentrations of both BLG and GMP in the binary mixture using only a spectrophotometer.

### 16.11.2 Results

The  $S_o$  for GMP did not differ significantly for the uncharged and positively charged membranes (Fig. 16.2). The positive charge on the membrane was more effective at retaining protein for BLG (Fig. 16.3). This was attributed to



**FIGURE 16.2** Observed sieving coefficient ( $S_o$ ) vs. flux for GMP using an ultrafiltration membrane that was either not charged (NC) or was charged positive (+), and the fit of Equation 12 to the data (calc). R1 and R2 are replicate experiments.



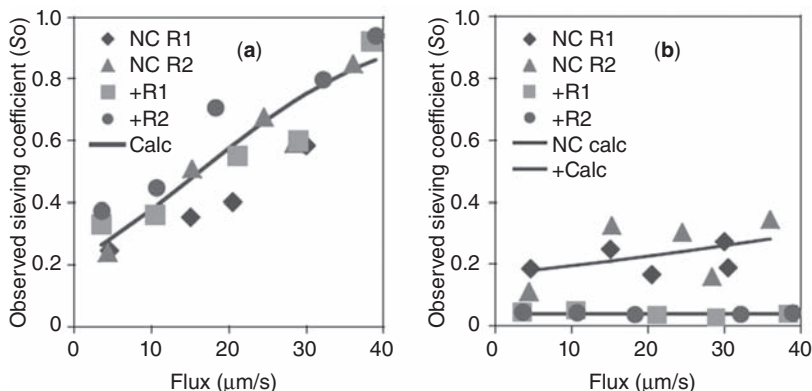
**FIGURE 16.3** Observed sieving coefficient ( $S_o$ ) vs. flux for BLG using an ultrafiltration membrane that was either not charged (NC) or charged positive (+), and the fit of Equation 12 to each data set (+calc and NC calc). R1 and R2 are replicate experiments.

the lack of charge on the acidic GMP ( $pI < 3.6$ ) at pH 3.0 compared to the strong positive charge on BLG ( $pI = 5.1$ ) at pH 3.0.

The observed sieving coefficient for GMP increased with increasing flux from  $S_o \approx 0.3$  to  $S_o \approx 1.0$  (Fig. 16.2). These results were expected based on the SFM and Equation 12. The  $S_o$  vs.  $J_v$  data for GMP were fitted using Equation 12, resulting in  $S_i = 0.23$  and  $k = 13 \mu\text{m/s}$ . The mass transfer coefficient calculated using Equation 7 was  $k = 2.1 \mu\text{m/s}$ . This difference is not surprising given that (i) the SFM is an approximation (5); (ii)  $S_i$  is not constant as assumed in the fitting procedure; it also depends on flux as shown in Equation 8; (iii) the correlation of Equation 7 is not perfect, and it is difficult to estimate the values of some of the parameters (e.g.,  $D$  and  $\nu$ ). Nevertheless, the SFM was in semi-quantitative agreement with the experimental data for GMP.

The  $S_o$  value for BLG did not depend as strongly on flux as it did for GMP, and was not a function of flux at all for the positively charged membrane (Fig. 16.3). The  $S_o$  vs.  $J_v$  data for BLG and the uncharged membrane were fitted using Equation 12, resulting in  $S_i = 0.25$  and  $k = 54 \mu\text{m/s}$ . The values of  $k$  for GMP and BLG should be about the same in Equation 12, but this was not the case. As a result, the shape of the plot of  $S_o$  vs.  $J_v$  was dissimilar for GMP and BLG.

Binary mixtures of proteins are not covered by the theories outlined above, only individual pure proteins in buffer. For binary systems, the more permeable protein must pass through a layer of the less permeable protein that builds up at the surface of the membrane. For that reason alone, the sieving coefficient is not simply a property of the membrane and the more permeable protein. Nevertheless, when the GMP and BLG system was observed as a mixture, the results were similar to the single proteins alone (Fig. 16.4). The data for  $S_o$  vs. flux for GMP (Fig. 16.4a) were fitted using Equation 12, resulting in  $S_i = 0.21$  and  $k = 12 \mu\text{m/s}$ , essentially identical to the single protein values



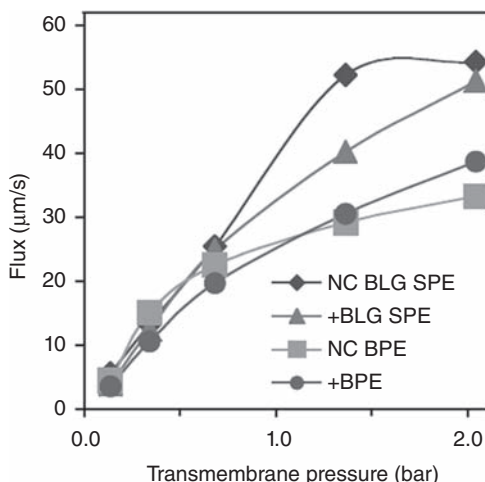
**FIGURE 16.4** Observed sieving coefficient ( $S_o$ ) vs. flux for a mixture of GMP and BLG using an ultrafiltration membrane that was either not charged (NC) or charged positive (+), and the fit of Equation 12 to each data set (+calc and NC calc). R1 and R2 are replicate experiments. (a) GMP and (b) BLG.

for GMP stated above. Similarly, the data for  $S_o$  vs. flux for BLG (Fig. 16.4b) were fitted using Equation 12, resulting in  $S_i = 0.17$  and  $k = 54 \mu\text{m/s}$ , essentially identical to the single protein values for BLG stated above.

Flux was a critical control variable for the fractionation of GMP and BLG as mentioned earlier. For example, using the positively charged membrane and the binary protein system, the selectivity of the separation ( $\Psi = S_{o,\text{GMP}}/S_{o,\text{BLG}}$ ) increased from  $\Psi = 7$  at  $J_v = 4 \mu\text{m/s}$  to  $\Psi = 22$  at  $J_v = 40 \mu\text{m/s}$ . By comparison, using the uncharged membrane and the binary protein system, the selectivity was much lower and depended less on flux:  $\Psi = 2$  at  $J_v = 4 \mu\text{m/s}$  and  $\Psi = 3$  at  $J_v = 40 \mu\text{m/s}$ . Thus, increasing flux increased the selectivity for the charged membrane more than for the uncharged membrane, and placing a positive charge on the ultrafiltration membrane increased selectivity. At the highest flux measured, the selectivity increased by over 600%.

Initially, flux increased linearly as  $\Delta P$  increased, but later the flux approached an asymptotic value (Fig. 16.5). According to Equation 5, flux is a function of the difference between the transmembrane pressure ( $\Delta P$ ) and the osmotic pressure ( $\Delta\pi$ ). Increases in the flux caused by increasing  $\Delta P$  are counterbalanced by increases in  $\Delta\pi$ . Experimental observations of flux vs.  $\Delta P$  confirmed these theoretical predictions. In addition, flux did not depend on membrane charge. In both single protein experiments using BLG, and binary protein experiments using a mixture of BLG and GMP, the flux vs.  $\Delta P$  response was the same for the uncharged and positively charged membranes. Thus, placing a charge on the membrane did not alter the membrane permeability and was not the reason for the measured differences in the observed sieving coefficient between charged and uncharged membranes. The flux was lower for the binary protein experiments compared with the single protein experiments simply because the protein concentration was greater in the former case.





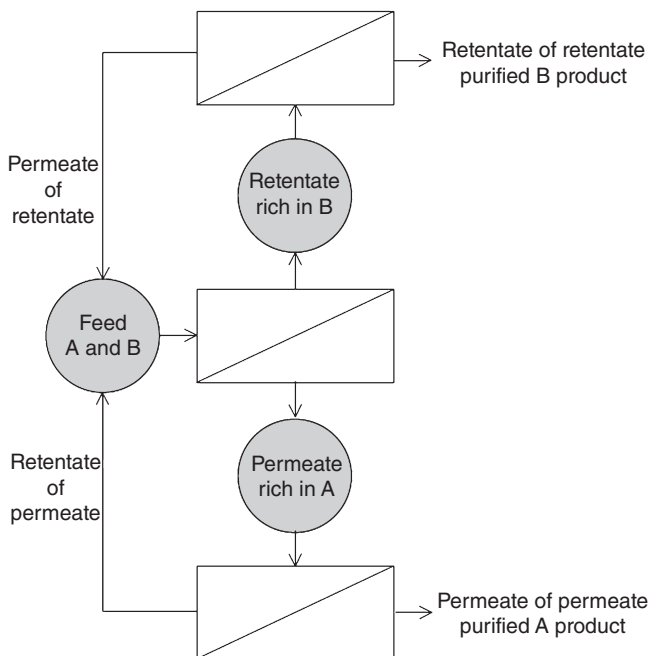
**FIGURE 16.5** Flux vs. transmembrane pressure for BLG alone, or a mixture of GMP and BLG, both using an ultrafiltration membrane that was either not charged (NC) or charged positive (+). SPE = single protein experiment; BPE = binary protein experiment.

### 16.11.3 Discussion

The advantages of charged ultrafiltration membranes are spelled out in this case study. Adding a positive charge to the membrane did not lower the flux, and increased the selectivity of the separation by over 600%. Whereas the two proteins of similar size could not be separated well using an uncharged membrane, the separation was very successful using the charged membrane. Therefore, one application enabled by charged membranes is the fractionation of similarly sized proteins, something that has not been feasible in the past using uncharged ultrafiltration membranes. Another application is to open up the pore size of the membrane to increase flux while maintaining the sieving coefficient at a low value. For example, in this case study, a positively charged membrane with a cutoff greater than 30 kDa could have been used to increase flux, because adding a charge to the membrane decreased the sieving coefficient for BLG by about sixfold. Using a charged ultrafiltration membrane of larger pore size than 30 kDa would achieve a much higher flux for the same retention of BLG.

## 16.12 MEMBRANE CASCADES

The advent of charged ultrafiltration membranes has enabled the fractionation of proteins, a unit operation typically reserved for chromatography. Lightfoot (14, 15) asks whether membrane cascades could replace chromatography.



**FIGURE 16.6** Schematic diagram of a three-stage membrane cascade for the separation of a feedstream containing two proteins, A and B.

Countercurrent stage operations have found great success in liquid–liquid extraction, distillation, and membrane separations. Lightfoot points out that uranium isotope separation in the 1940s used membrane cascades, and the theory was spelled out at that time, but was never applied to the biotechnology industry. He argues that because membrane selectivity is increasing, and cascades can further increase that selectivity, then chromatography could be replaced by membrane separations.

As a first implementation, batch operation using three steps has been recommended (16) (Fig. 16.6). First, the feedstream containing Proteins A and B is fractionated into a permeate stream rich in A and a retentate stream rich in B. Next, the retentate stream rich in B is processed into a second permeate stream that is sent back to the feedstream, and into a second retentate stream that is the purified Protein B product. Next, the permeate stream rich in A is processed into a third permeate stream that is the purified Protein A product, and into a third retentate stream that is sent back to the feedstream. The two streams sent back to the feedstream have about the same ratio of the two proteins, A and B, as the feedstream. Three membrane units are shown in Fig. 16.6 for illustration purposes only. In practice, only one membrane unit would exist, and it would be used three times. Although complicated, the advantages of continuous operation and a forgiving, operator-friendly process for ultrafil-

tration compared to chromatography may justify the use of simple membrane cascades in the future.

### 16.13 CHARGED MICROFILTRATION MEMBRANES

Charged microfiltration membranes work by protein adsorption as opposed to charged ultrafiltration membranes where the charge serves to decrease the permeation rate of proteins. Charged microfiltration membranes were introduced as a product in 1988 as a means to overcome the limitations of column chromatography. Charged microfiltration membrane products are also known as membrane chromatography, adsorptive membrane, and membrane adsorber products. In all cases, the chromatographic media consist of microporous membranes containing an immobilized adsorptive ligand. Pressure drop limitations are not significant because the membranes are thin ( $\sim 100\mu\text{m}$ ). Solute is transported through the pores of the membrane by convection, not diffusion, which eliminates diffusional limitations. Although the first products were hollow fiber membranes, where the surface was activated for affinity ligand attachment, most products now use IEX as the primary ligand type. Initially promoted for protein purification, the primary target now is the purification of large biomolecules such as plasmid DNA, viruses, and very large proteins ( $>250\text{kDa}$ ) where chromatography beads have low dynamic capacity. Three vendors have dominated the manufacture of adsorptive membrane products: Millipore Corporation (Bedford, MA, USA), Pall Biopharmaceuticals (East Hills, NY, USA; Mustang<sup>TM</sup>), and Sartorius Stedim AG (Goettingen, Germany; Sartobind<sup>TM</sup>).

A technology with similar advantages is monolithic chromatography from BIA Separations (Ljubljana, Slovenia; Convective Interaction Media<sup>TM</sup>). Monoliths are not membranes at all, but do share the features of convective transport, low pressure drop, high dynamic capacity, and elimination of the diffusion limitation to adsorption found using beads. The principles and applications discussed in this section apply to both technologies. This section will focus on the separation of large biomolecules such as viruses for removal of biological impurities from mAb products.

### 16.14 VIRUS CLEARANCE

Virus clearance is essential in the manufacture of biotechnology-derived products such as mAbs (17) (see Chapter 8). Regulatory agencies worldwide, including the Food and Drug Administration (FDA), require that freedom from virus contamination is demonstrated before new biopharmaceutical products are approved for human use (18, 19). Key components include specific virus removal steps such as filtration and small-scale studies that measure their clearance capacity and robustness.

The nanometer-scale size of virus particles makes separation from biopharmaceutical process intermediates a challenging manufacturing issue. Virus particles bind only to the surface of traditional chromatography beads because they are too large to enter the fine network of pores (20–22). Therefore, the binding capacity for virus particles is much lower than it is for smaller molecules, which are able to access the full volume of the beads. This phenomenon causes a problem: the binding capacity is much greater for small impurities, HCP, and endotoxin than for the large target, the virus particle (23).

As discussed for beads, virus particles bind only to the surface of membranes, but membranes have a larger available surface area than beads. For example, microporous membranes have an internal surface area of  $\sim 1.1 \text{ m}^2/\text{mL}$  (24), compared to about  $0.11 \text{ m}^2/\text{mL}$  for a column packed with  $90\text{-}\mu\text{m}$  beads. Furthermore, the adsorptive capacity of membranes increases with increasing size of the adsorbed particle, because larger particles form a thicker layer on the membrane surface (20). The net effect is that adsorptive membranes have a high capacity for large nanometer-sized particles and have a low capacity for small molecules (25), exactly the opposite of the situation with beads. Thus, the relative advantage of membranes over beads increases dramatically as the particle size increases, making adsorptive membranes well suited for virus clearance.

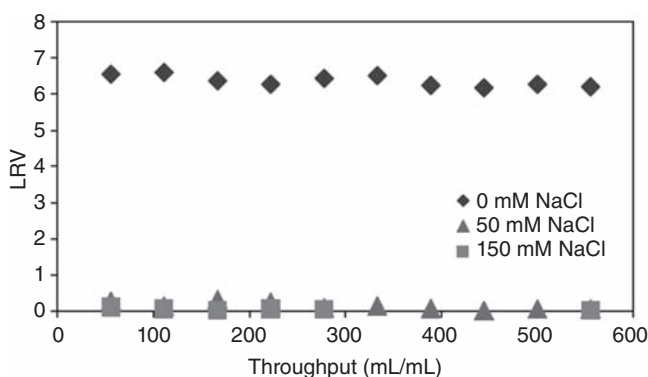
Traditional chromatography beads were designed for protein, not virus separations (22). Adsorptive membranes have the advantage of low cost, low volume, and disposability. Beads were designed to be reused, and process economics encourages their use for hundreds of cycles (26). Regeneration is therefore essential, meaning that ligands must bind their target reversibly. Resin cleaning and lifetime validation costs are considerable for beads. These restrictions are absent for adsorptive membranes because they are disposable and do not need to bind the virus reversibly for clearance applications, making new tighter-binding ligands practical. The higher dynamic capacity of adsorptive membranes reduces the adsorbent volume, requiring smaller buffer volumes, lower consumption of pharmaceutical grade water, and less floor space for buffer tanks and pumps, all of which lead to reduced facility costs, a major expense for bioprocessing. The pass-through-and-dispose operational mode also reduces the required equipment space compared to chromatography columns, eliminating the need for dedicated rooms for this unit operation.

Robust, uniform, and predictable virus clearance by membrane adsorbers enables generic and bracketed validation strategies (27). The FDA defines a generic clearance study as one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model antibody, and when these data may be extrapolated to other antibodies following the same procedure. Bracketing is where an applicant demonstrates virus removal/inactivation for a particular module at two different values of a given parameter (e.g., ionic strength, dwell time, temperature) and may use any values of that parameter falling within that range. These concepts presaged the design

space concept proposed recently by the International Conference for Harmonization as part of the Quality by Design initiative for pharmaceuticals (ICH Q8) (28). Generic and bracketed validation strategies are even more powerful as they can be applied across products. Examples of two matrix/bracket studies of robust virus clearance steps [e.g., low-pH inactivation and anion-exchange (AEX) chromatography] have been described in the literature (29,30). Bracketing and generic validation was proposed by the FDA to streamline clinical trial stage mAb development by eliminating redundant testing, conferring flexibility during product development, and spurring product development.

### 16.15 SALT TOLERANCE

All existing adsorptive membranes use a quaternary amine ligand to bind viruses. Therefore, one problem with virus clearance using adsorptive membranes is that existing products cannot remove neutral viruses from feed solutions with even moderate salt concentrations. The Mustang Q product (Acrodisc® 25 mm, Pall Life Sciences, Ann Arbor, MI, USA) is a good example. Using this membrane, a neutral virus ( $\phi$ X174, pI = 6.6) can be cleared from a salt-free feed solution at pH 7.5 with a log reduction value (LRV) of 7. However, in the presence of 50 mM NaCl, the virus passes freely through the adsorptive membrane into the product and the LRV falls essentially to zero (Fig. 16.7). The neutral virus  $\phi$ X174 is one of five model nonpathogenic bacteriophage viruses commonly used in filtration studies to represent the gamut of physical properties of potential adventitious viruses that are hazardous to humans (e.g., size, pI, presence or absence of a membrane). The current virus safety assurance strategy for biopharmaceuticals should preclude the presence



**FIGURE 16.7** Log reduction value (LRV) vs. throughput (milliliters of feed solution per milliliter of membrane volume) for clearance of the neutral virus  $\phi$ X-174 using a Mustang™ Q membrane adsorber for different salt concentrations in TE buffer, pH 7.5.

of viruses in the feed solution, and careful control of cell lines and raw materials should prevent virus introduction into the manufacturing process (31). However, viral contamination in bioprocessing is essentially stochastic, e.g., from contaminated raw materials. Thus, it is impossible to predict which virus could be next introduced into a manufacturing process. Therefore, one has to be prepared to remove all viruses, even neutral viruses. The existing adsorptive membrane products cannot do that.

Salt tolerance is important for the design of robust virus clearance technologies because many process solutions used in biopharmaceutical manufacture must have conductivities of 15–30 mS/cm to minimize product aggregation. Salt tolerance is measured in comparison to the traditional Q ligand [2-aminoethyltrimethylammonium chloride (AETMA)], which rapidly loses capacity for some viruses (e.g.,  $\phi$ X174) at conductivities of three- to sixfold less than the target range. Using the Q adsorptive PVDF membrane from Millipore, the clearance of  $\phi$ X174 dropped from an LRV of 6 with no salt to less than 1 in 50 mM NaCl (32). We found the same behavior using regenerated cellulose membranes containing the same Q ligand (Table 16.1). The Q ligand is a frequent choice for AEX chromatography for virus clearance operations (30, 33).

In our recent research, two salt-tolerant ligands were examined using two function tests (34). In the first function test, bovine serum albumin (BSA) in 20 mM piperazine, pH 6.0, was incubated with a functionalized regenerated cellulose membrane with and without added NaCl, and bound BSA was measured using the bicinchoninic acid (BCA) colorimetric method. Function test 1 measured the static protein binding capacity of the membranes under conditions where the pH was close to the pI of the protein (BSA pI = 5.1). In this

**TABLE 16.1 Performance of Ligands Immobilized onto Regenerated Cellulose Membranes**

Ligand	NaCl, mM	BSA static capacity, mg/m <sup>2</sup>	LRV <sub>avg</sub>
2-Aminoethyltrimethylammonium chloride (AETMA, Q ligand)	0	551	6.6
	50	42	0.05
	150	10	0.05
RC-1 (Tris(2-aminoethyl)amine)	0	929	6.2
	50	402	5.4
	150	136	5.4
RC-3 (agmatine)	0	1000	5.5
	50	319	5.5
	150	116	5.9
Blank	0	19	0.07
	50	26	0.06
	150	19	0.05

*Note:* RC-1 and RC-3 are salt-tolerant ligands, and blank is no ligand.

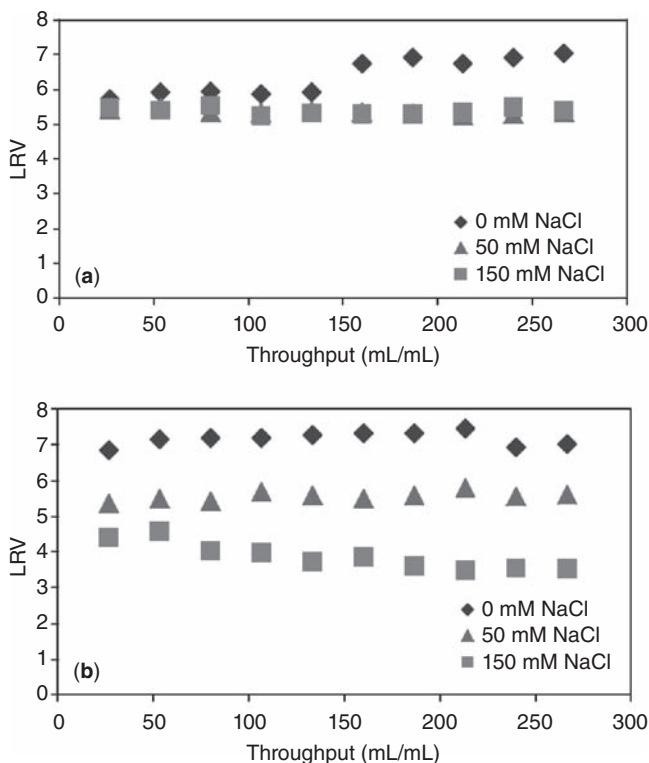
function test, the protein had only a weak charge and was sensitive to added salt. This test was designed to mimic the binding of a neutral virus where the charge was weak. Function test 2 measured the LRV for the candidate virus and the functionalized membrane in flow-through mode (six layers of membrane) using TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) with and without added NaCl. Each ligand was immobilized onto 0.45- $\mu$ m pore regenerated cellulose membranes (Type 184, 25-mm diameter, Sartorius Stedim Biotech) using allyl glycidyl ether followed by bromination of the allylated membrane and then coupling to the primary amine on the ligand.

The performance of the two salt-tolerant ligands was superior to the traditional Q ligand (Table 16.1). The Q ligand retained only 2% of the BSA static capacity and none of the virus clearance capacity when the salt concentration was increased from 0 to 150 mM NaCl. In contrast, ligands RC-1 and RC-3 retained 15% and 12% of the BSA static capacity, and 90% and 100% of the virus clearance capacity, respectively, with increased salt concentration. The virus clearance capacity was not reduced by the addition of more virus into the membranes (Figs. 16.8a and 16.9a).

Is the virus clearance capacity for the two salt-tolerant ligands maintained in the presence of mAb? To answer this question, 0.5 g/L of ribonuclease A (RNase A) was added to the virus feed solution. RNase A is an antibody mimic in this case because it has a pI of 9.5 making it positively charged at pH 7.5. Most therapeutic mAbs tend to have pIs between 8 and 10. This narrow pI range exists because therapeutic mAbs tend to be human IgG1, or to a much lesser extent IgG2 and 4. Because the V regions of an antibody represent a small percentage of the total molecule, charge is largely determined by isotype. Thus, mAbs are positively charged at neutral pH, preventing binding to AEX media (30). Adding the mAb mimic to the feed solution did not reduce virus clearance (Figs. 16.8b and 16.9b). Thus, new membrane adsorber products that achieve salt-tolerant virus clearance for mAb products are likely to be developed using new ligands.

## 16.16 CONCLUSIONS

This chapter has illustrated the utility of adding a charge to the surface of existing ultrafiltration and microfiltration membranes. The separation power of these membrane products is greatly increased by this modification. Ultrafiltration membranes could separate proteins of similar size when a charge was added to the membrane, increasing the selectivity of the separation by over 600% without any decrease in membrane flux. The use of membrane cascades may further enhance the fractionation of proteins using charged ultrafiltration membranes. Adding a charge to microfiltration membranes facilitated virus clearance without affecting mAbs in the same feed. Over 99.99999% of virus was removed from the feed while the mAb mimic passed freely through the membrane. New salt-tolerant ligand chemistries were shown to trap neutral



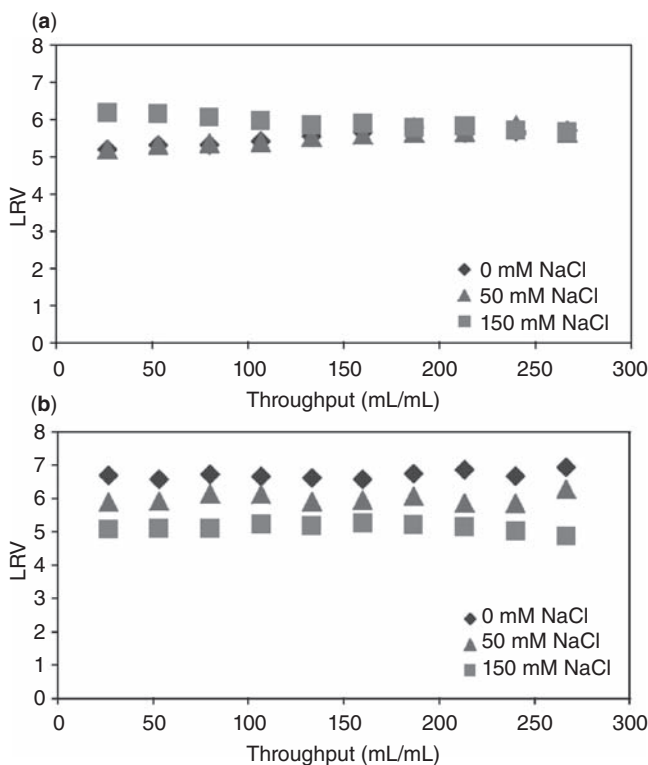
**FIGURE 16.8** Log reduction value (LRV) vs. throughput (milliliters of feed solution per milliliter of membrane volume) for clearance of the neutral virus  $\phi$ X-174 using regenerated cellulose membranes and the immobilized salt-tolerant ligand RC-1 for different salt concentrations in TE buffer, pH 7.5. (a) Without RNase A and (b) with added RNase A.

viruses in the presence of up to 150 mM NaCl. These examples demonstrate that dramatic advances in the usefulness of ultrafiltration and microfiltration membrane separations are possible merely by adding a charge. We hope this chapter inspires the increased use of charged membrane technology for the process-scale purification of mAb products.

## 16.17 ACKNOWLEDGMENTS

The author is grateful to Kurt Brorson and Scott Lute at the FDA for all their assistance in the form of patient training and education on the work related to virus clearance using membrane adsorbers. Without their help, the author would still think of clearance as a half-off sale at a department store. Bill Riordan and Shanti Bhushan are PhD students who worked in the author's





**FIGURE 16.9** Log reduction value (LRV) vs. throughput (milliliters of feed solution per milliliter of membrane volume) for clearance of the neutral virus  $\phi$ X-174 using regenerated cellulose membranes and the immobilized salt-tolerant ligand RC-3 for different salt concentrations in TE buffer, pH 7.5. (a) Without RNase A and (b) with added RNase A.

laboratory and obtained all the original data presented in this chapter. Funding for the work on charged ultrafiltration membranes was from Dairy Management Inc. The National Science Foundation and an industrial sponsor funded the work on membrane adsorbers.

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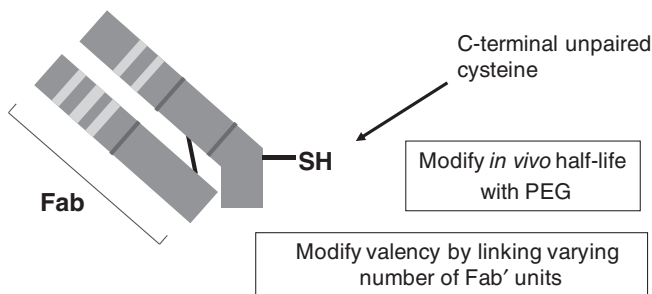
## DOWNSTREAM PROCESSING OF MONOCLONAL ANTIBODY FRAGMENTS

MARIANGELA SPITALI

### 17.1 INTRODUCTION

Antibody fragments such as the fragment antigen binding (Fab) and the single-chain fragment variable (scFv) are traditionally considered as therapeutic formats for the treatment of acute indications. Examples include ReoPro (a chimeric Fab produced in mammalian cells for the treatment of cardiovascular disease) and polyclonal antibody fragments such as CroFab (which is used as an antidote to rattlesnake venom), DigiFab and Digibind (which are used to treat digoxin overdose). Antibody fragments have also proven useful as diagnostic tools, e.g., arcitumomab, the Food and Drug Administration (FDA)-approved Fab' fragment used to image colorectal cancer, and CC49, a scFv currently in development used to image gastrointestinal malignancies in preparation for radioimmunotherapy. More recently, antibody fragments produced in microbes, such as Lucentis [an antivascular endothelial growth factor (VEGF) Fab approved for the treatment of age-related macular degeneration], have also been used in acute clinical settings.

Fab' fragments, which include a hinge region, offer greater potential avidity. As indicated in Fig. 17.1, a single cysteine residue has been engineered in the hinge to allow the formation of di- or tri-Fab' entities (1). The unpaired hinge thiol can also provide an attachment point for site-directed polymerization to extend the half-life of the antibody, making it suitable for therapies requiring



**FIGURE 17.1** Antibody Fab' fragments are considered a flexible building block, engineered with a C-terminal unpaired cysteine in the hinge region for site-directed PEGylation to modify half-life *in vivo* and also to tailor valency such as mono-, di-, and trivalent species formats.

prolonged activity (2, 3). As a safety consideration, another advantage of Fab' fragments is the lack of toxicity normally associated with the fragment-crystallizable (Fc) portion of the antibody, making such product formats necessary for certain clinical indications. Modification of antibody fragments through the covalent attachment of large polymers such as polyethylene glycol (PEG) or human serum albumin helps to tailor the half-life of the molecules for specific clinical requirements, enabling the treatment of chronic indications requiring extended pharmacokinetic profiles *in vivo*. For example, certolizumab pegol, which is used to treat chronic and relapsing Crohn's disease and rheumatoid arthritis, is a humanized Fab' fragment expressed in *Escherichia coli* and modified by site-specific PEGylation. CDP791 is a humanized fragment under development at Union Chimique Belge (UCB) that is used to block the VEGF- $\beta$  receptor and is modified using a *bis*-maleimide PEG species, resulting in a divalent PEGylated Fab' product that has the correct characteristics for blocking angiogenesis, thus leading to the reduction of solid tumors.

Designing novel antibody-based therapeutic formats means exploiting their very selective and high-affinity characteristics. Various recombinant antibody fragment formats have therefore been engineered to provide a more flexible approach to fulfill unmet needs in the clinic. With more than 30 such products either approved, in the market, or currently in clinical development (4), it is evident that antibody fragments are a viable, safe, and often necessary alternative to whole monoclonal antibodies (mAbs). Recently engineered structures which include single domain antibodies (dAbs), nanobodies, minibodies, and bispecific constructs [such as r28M, a bispecific scFv targeted to CD28 and MAP used for the treatment of melanoma (4)] are emerging as alternative therapeutic entities. The smaller species format improves transport into specific tissues, including tumor penetration. Antibody fragments should also be considered as a viable option for clinical central nervous system (CNS) indications, as smaller and specifically tailored products are designed to cross the blood–brain barrier efficiently.

## 17.2 PRODUCTION OF ANTIBODY FRAGMENTS FOR THERAPEUTIC USE

Antibody fragments can be easier to produce than whole mAbs. Nonglycosylated fragments can be expressed using rapid and simple fed-batch microbial fermentation with chemically defined media, in contrast to the more complex mammalian systems that are necessary for the production of glycosylated, full-size mAbs (5–7). In chronic and relapsing conditions, repeat dosing is often required in order to treat patients over several years, making production and availability a growing concern. Fab' fragments such as certolizumab pegol, which lack the Fc portion, are not glycosylated and can therefore be expressed in *E. coli* by fermentation, provide a relatively simple, rapid, reproducible process and a consistent, reliable supply of the product. Purified yields of more than 500 mg/L are possible for many Fab' species and scale-up to 12,000 L has been achieved for certolizumab pegol (8).

Mammalian cell culture technology is currently limited to the 25,000-L scale, whereas fermentation technology for bacteria and yeast can be carried out at much larger scales. The economies of scale and rapid process times associated with microbial fermentation strategies create an effective system for the production of therapeutic proteins, making microbial production suitable for the annual production requirements of large market indications. Unlike mammalian expression systems, uniformity of expression among microbial clones is ensured because the transgenes are contained on an extrachromosomally replicating plasmid. For all these reasons, a variety of antibody fragments and conjugates have been developed to address specific indications, and their production is facilitated through the use of low-cost, microbial expression systems.

## 17.3 DOWNSTREAM PROCESSING

Biopharmaceuticals suitable for administration to humans must meet very high standards of purity. Throughout downstream processing, it is important to ensure that the integrity and biological activity of the target protein are retained, while reducing all product- and process-related impurities and additives to acceptable levels. In order to do this, good knowledge of the product's impurity and stability profile must be gathered as early as possible so that effective process steps and parameters are built into the process, making it fully integrated and able to achieve the final product quality required for use in the clinic. An understanding of where potential product impurities can be generated during manufacture should be sought, so that they can be controlled appropriately and removed during subsequent processing steps.

Downstream processing of recombinant antibody fragments can be divided into four areas: (i) initial isolation of the soluble product from the host cell; (ii) primary capture to stabilize and concentrate the target protein and to

remove major host cell impurities; (iii) removal of residual host cell impurities; and (iv) one or more polishing steps to separate the product from any product-related impurities, ensuring it is suitable for its intended use in preclinical and clinical studies, with the ultimate aim of developing an effective, economical, and robust commercial process.

In addition, for recombinant products expressed in mammalian cell culture systems, at least two virus clearance measures must be included, such as low-pH inactivation and a virus filtration step. Another advantage of microbial expression systems is that virus inactivation or removal is unnecessary, and the expensive and complicated virus clearance validation studies and continual quality control testing for virus elimination are therefore not required.

### 17.3.1 Primary Recovery

Antibody fragments are usually engineered for intracellular expression rather than secretion into the culture supernatant. Therefore, industrial-scale extraction involves disruption of the host cells by lysis using either chemical or mechanical disruption techniques. The cytoplasmic extract then needs to be submitted to a series of unfolding and refolding techniques to ensure the product is fully soluble and correctly folded. In gram-negative microbial systems, where the product can be targeted to the periplasmic space between the inner and outer cell membranes, the outer membrane can be selectively disrupted to release the product, keeping the cytoplasm intact and limiting the amount of host cell protein (HCP) in the extract. Disruption is usually followed by removal of cells and/or cell debris to obtain clarified feedstock for subsequent processing steps.

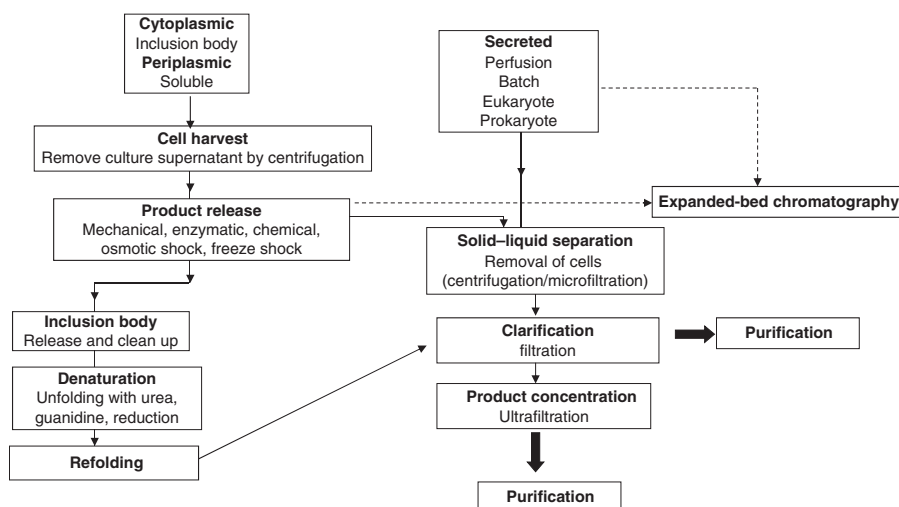
This approach differs from antibody expression systems in which the product is secreted into the cell culture supernatant, where primary recovery would simply involve the removal of cells by centrifugation or microfiltration. In addition, the extracellular media from fermentation cultures contain relatively few proteins, and this simplifies the downstream processing. However, some products are unable to withstand the high shear forces generated by microbial fermentation (9) and the yields are lower if such proteins are secreted. Harsh conditions at the bioreactor air/liquid interface, including the release of proteases, may also lead to product degradation or aggregation. In contrast, intracellular products exist in a more protective environment leading to better control and fewer product-related impurities.

Expression in the cytoplasm is often considered disadvantageous in a therapeutic setting, e.g., in *E. coli* where the cytoplasm is a reducing environment that prevents the formation of disulfide bonds. Appropriate refolding of antibody fragments, such as Fab' species, has yet to be demonstrated, raising concerns over batch-to-batch consistency. However, the expression of such therapeutic antibody fragments in inclusion bodies within the cytoplasm should not be overlooked, as very high levels of protein expression are possible. In combination with the short fermentation time and the potential for

simple and high-yielding primary purification steps, inclusion bodies offer an attractive manufacturing option. Targeting proteins to the periplasm is also advantageous (see above) because this compartment favors the formation of disulfide bonds and allows more complex antibody fragments to be expressed. Within the *E. coli* periplasm, antibody fragments are able to fold into the native state and can be recovered using simple extraction procedures. Periplasmic expression levels as high as 1–2 g/L have been reported for antibody fragments during high-cell-density fermentation (10). However, the level of expression can vary considerably and is largely dependent upon the primary sequence of the protein.

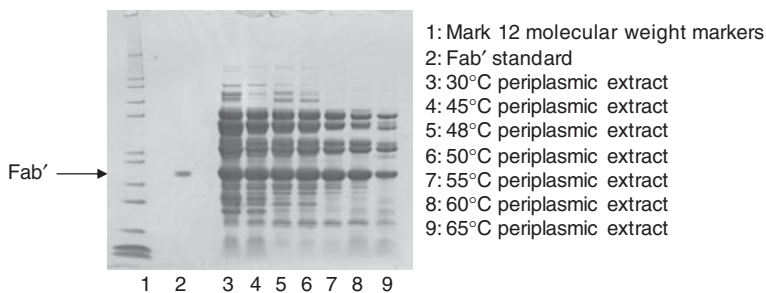
Primary recovery procedures often perform one or more functions, such as separation of cells or particulate matter from liquid broth, product concentration, and feedstream conditioning in preparation for the primary capture step. Feedstreams differ considerably according to the production method, so different strategies are required for primary recovery as shown in Fig. 17.2. Typically, cells and particulate matter are separated from conventional fermentation broths by centrifugation or microfiltration, followed by depth and/or dead-end filtration to exclude particulates greater than 0.22  $\mu\text{m}$  in diameter, an absolute requirement to ensure the product stream is appropriately treated prior to entering environmentally controlled downstream processing suites.

Following cell culture fermentation, the product stream fraction chosen for further processing depends on whether the product is cell associated or secreted. Cell-associated material must be released by an extraction step. For an intracellular product, this can be achieved using a homogenizer for cell disruption, which often results in an undesirable and complex mixture of



**FIGURE 17.2** Industrial methods used for the primary capture of biopharmaceuticals in preparation for purification.

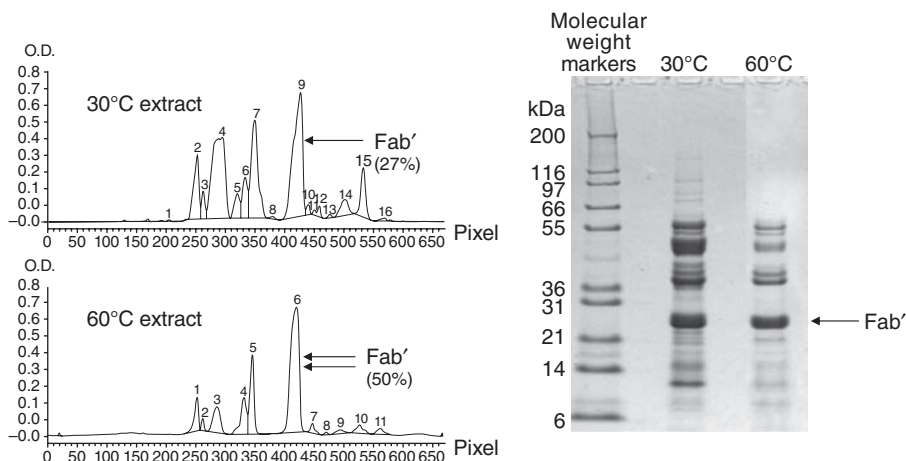




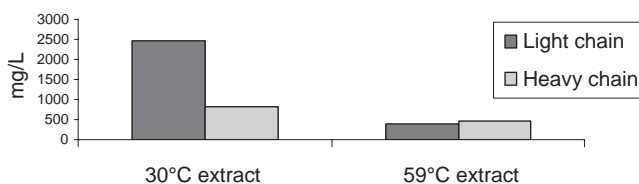
**FIGURE 17.3** Reducing SDS-PAGE analysis, with Coomassie blue staining, demonstrating the effect of various heat treatments (30–65°C) during Tris/EDTA periplasmic extraction of a recombinant humanized Fab' fragment produced by *E. coli* expression.

proteins for subsequent purification steps. However, as stated above, the release of antibody fragments from the *E. coli* periplasm can be achieved by methods that disrupt the outer cell membrane and maintain inner membrane integrity, e.g., osmotic shock, Tris buffer, and ethylenediaminetetraacetic acid (EDTA) treatments (11). The *E. coli* periplasm contains significantly fewer proteins than the cytoplasm, so the antibody fragment of interest is effectively concentrated, making its purification less complicated. Initial purification can be enhanced further by the inclusion of a heat-treatment step (12). At elevated temperatures (>40°C), correctly assembled antibodies and antibody fragments are remarkably stable, whereas many other proteins in the feedstock form precipitates and/or aggregates, which can easily be removed during centrifugation, filtration, or primary capture.

Figure 17.3 shows the advantages of applying heat to reduce HCP and of optimizing the temperature to ensure yield is not compromised. Product quality can be improved significantly with up to 50% of HCP being removed by heating (Fig. 17.4). Furthermore, truncated and incorrectly folded product species can also be removed by the inclusion of a heat-treatment step during the extraction process. Figure 17.5 shows that the inclusion of a heat-treatment step can reduce the levels of free antibody chains as detected by the Biacore™ surface plasmon resonance assay, implemented to measure total heavy (Fd) chain and kappa light chain in a periplasmic cell extract. This is an excellent example of a product stream containing an abundance of free chains, which are labile to the heat treatment, resulting in the appropriate ratio of Fd and light chains following the heat-treatment step. It should also be noted that Fd and light chains that are not joined by interchain disulfide bonds are usually paired in solution through hydrophobic and ionic interactions, and therefore such species would be difficult to separate from the correctly paired parent molecule during subsequent processing steps. It is for these reasons that the inclusion of a pretreatment step such as heat treatment is recommended during primary recovery. Such methods can be valuable tools,



**FIGURE 17.4** Comparison of 30 and 60°C periplasmic cell extract of a microbially expressed recombinant humanized Fab' fragment. Analysis was performed by densitometry of Coomassie blue-stained SDS-PAGE run under reducing conditions.

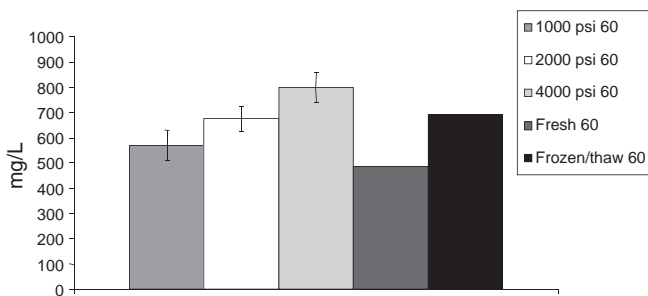


**FIGURE 17.5** Assessment of the ratio of expressed Fab' Fd (heavy chain) and kappa light chain using Biacore assay to compare non-heat-treated periplasmic extracts (30°C) with heat-treated (59°C) periplasmic extracts.

ensuring the appropriate product quality is achieved, as well as streamlining the subsequent processing steps.

Additional yield improvement techniques for antibodies expressed in the bacterial periplasm include low-pressure homogenization (13) and freeze-thawing (14). When coupled with a heat-treatment step, product titers can be improved without cell lysis (Fig. 17.6). Implementation of yield improvement techniques, such as mild homogenization, generates a more homogenous product stream by gentle declumping of cell clusters and cell aggregates, making the cell surface more accessible to buffer components required for the extraction process.

Following primary recovery, some conditioning of the feedstream may be required in preparation for the first purification step. For example, adjustments in pH or ionic strength may be required to promote binding between the target protein and the capture adsorbent. These may present challenges, particularly where large volumes need to be conditioned, possibly requiring large tanks for dilution adjustments, in-line dilution, or diafiltration steps.



**FIGURE 17.6** Histogram demonstrating the effect of mild homogenization (1000, 2000, and 4000 psi) applied to precondition *E. coli* cells prior to periplasmic extraction with Tris/EDTA, coupled with a 60°C heat-treatment step, to release recombinant humanized Fab'. The preconditioning homogenization treatment was compared to nonhomogenized cells (fresh) and frozen/thawed cells.

### 17.3.2 Capture

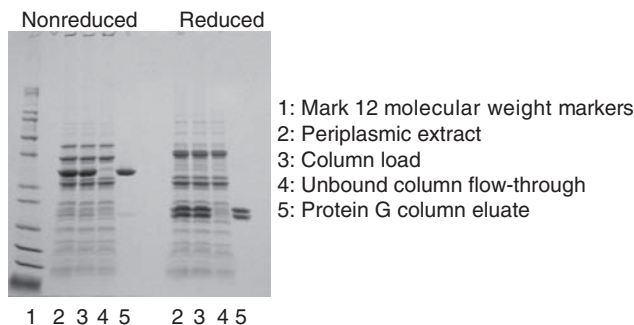
Primary capture of the target protein usually involves a chromatography step that concentrates the product from large feedstream volumes used at the manufacturing scale, as well as reduces the levels of the major process-related impurities. As a general rule, the capture step should be as selective as possible, but high-resolution resins are not necessarily required at this stage of the manufacturing process. mAbs are generally captured by very selective affinity chromatography resins based on Protein A. However, affinity capture may not be the best route for antibody fragments and single dAbs, due to either poor binding resulting in low dynamic binding capacities or the need for alternative, expensive resins that are not feasible or economical for large-scale applications. Consequently, other capture modes are often used, including ion-exchange (IEX) and hydrophobic interaction chromatography (HIC), thiophilic resins, and mixed-mode chromatography. Alternatives to conventional chromatography techniques, such as “salting out” (e.g., ammonium sulfate precipitation at low temperatures), could be used to concentrate/dewater high-volume feedstreams while selectively removing some impurities. Although this may be a viable option for some applications, the results are often hampered by poor product recoveries and by the formation of aggregates.

The primary capture step is often required to concentrate the feedstream and to remove the major process-related impurities. Common process impurities, obviously dependent on the expression system, must be controlled either by reducing them to undetectable levels, or at least to acceptable and safe levels, or by inactivation. Such impurities may include HCP, DNA, endotoxins, viruses, and process additives such as antifoam, antibiotics, and chelators. In addition, product-related impurities such as aggregates, degradation products, deamidated species, adducts, C-terminal clipped variants, glycosylated variants,

glycoforms, and other product isomers must be controlled and maintained at acceptable and /or consistent levels.

Affinity purification using an immobilized antigen is particularly useful for small fragments such as single dAbs (15), Fv (fragment variable), and scFv formats, which are not readily purified by other affinity methods (16). The degree of purification can be very high, but the antigen can be very expensive, making this approach unsuitable for large-scale manufacturing processes. A variety of chromatographic techniques are used for the purification of antibody fragments, many of which are similar to those used for mAb purification. For example, some antibody fragments can also bind to low-affinity sites on Proteins A and G. Protein A binds to the  $V_H$  (variable heavy) region of Fabs belonging to human gene family subgroup 3 ( $V_{H3}$ ) and specifically to sequences in the second complementarity determining region (CDR) and framework regions 1 and 3 (17, 18). Consequently, recombinant humanized Fabs have been purified successfully using immobilized Protein A (10, 17, 19). Similarly, Protein G has been used to purify Fab' molecules (20, 21) as shown in Fig. 17.7, where the product has been captured using Protein G Sepharose, reducing HCP significantly in the first step. Structural studies have shown that Protein G interacts with the  $C_{H1}$  (constant heavy) domain of Fabs (22), but this interaction is relatively weak, resulting in low dynamic capacities. An alternative bacterial IgG-binding protein that can be used for antibody purification is Protein L derived from *Peptostreptococcus magnus*. Protein L binds specifically to light-chain variable regions belonging to the human gene families  $\kappa 1$ ,  $\lambda 2$ , and  $\lambda 3$ , but not  $\kappa 4$  or  $\lambda 1$  (17, 23).

For antibody fragments that bind relatively strongly to Proteins A, G, or L, the feedstock often requires little conditioning, making these ligands ideal for purifying antibodies from feedstreams that have low titers and high volumes. The bound product is usually eluted from the column by lowering the pH, which also constitutes a virus inactivation step (requiring a specified low-pH hold). However, some antibodies may not be stable at low pH, resulting in aggregation, precipitation, and product inactivation. For antibody fragments



**FIGURE 17.7** Protein G affinity chromatography of an *E. coli*-expressed humanized Fab' analyzed by SDS-PAGE under reducing and nonreducing conditions.

that bind relatively weakly to these ligands, the binding conditions can be made more stringent by the addition of salt. The addition of 1 M glycine or 4 M NaCl to the feedstream can improve the binding capacity of the resin. Antibody fragments tend to interact weakly with Protein G, leading to fast off rates, so the ratio of the column feed to the column bed volume should be kept lower than 5:1. This may be achieved by concentrating the feedstream by ultrafiltration prior to column loading, or by applying the product to the column in a number of cycles.

The limitations of affinity chromatography for the capture of antibody fragments include not only the high cost of the resins and cleaning reagents (for resins that are unstable in caustic solutions), but also the low affinity of the resins, which means that large bed volumes are required or repeat column cycling is necessary. Column cycling may suit longer upstream processes, such as the 10- to 14-d duration typical for Chinese hamster ovary (CHO) cells. However, for faster platforms such as *E. coli*, where the fermenter operates for only 2–3 d, repeat column cycling would cause a bottleneck in the manufacturing plant, increasing the batch cycle time. Column sanitization and cleaning is an absolute requirement under current good manufacturing practice (cGMP), yet some affinity resins require expensive cleaning solutions that may not achieve complete sanitization. Recently developed Protein A resins are stable in contact with NaOH, making these a good option to ensure thorough cleaning between batches, leading to improved batch-to-batch consistency.

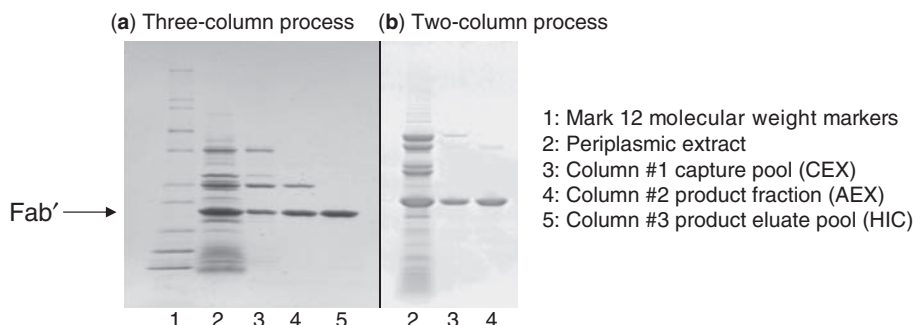
An alternative approach for the purification of antibody fragments is the use of affinity peptide tags that are engineered into the protein, usually at the C-terminus (the N-terminus may be preferred if C-terminal clipping is an issue), and are removed later by chemical or proteolytic cleavage. The hexahistidine (His<sub>6</sub>) tag is often used and facilitates purification by immobilized metal-ion affinity chromatography (IMAC). Histidine residues bind to immobilized nickel, copper, and zinc ions. The tagged protein is then eluted under gentle conditions using imidazole, which competes for metal-binding sites. A histidine-tagged scFv has been purified by IMAC and used in the clinic (24). Strep-tag, a biotin mimetic peptide that binds to immobilized streptavidin (25), and the FLAG tag, which is captured using an immobilized antibody (26), are alternative tags that can be used to purify small antibody fragments. Peptide tags are often cleaved by proteases such as Factor X, tobacco etch virus (TEV) protease, or thrombin for research preparations, but these are undesirable in a therapeutic setting since the production of high-grade proteases is costly. However, a number of chemical approaches have been developed, including cleavage by cyanogen bromide (27), *o*-iodobenzoic acid (28), and site-specific cleavage by Cu<sup>2+</sup> ions (29). Residual amino acids at the cleavage site can be an issue with all of these approaches, and may increase the likelihood of immunogenicity.

New synthetic ligands that are cheaper than protein-based affinity ligands and are more robust under stringent conditions provide a suitable alternative to Protein A. Peptide ligands, which reversibly bind antibodies or mimic

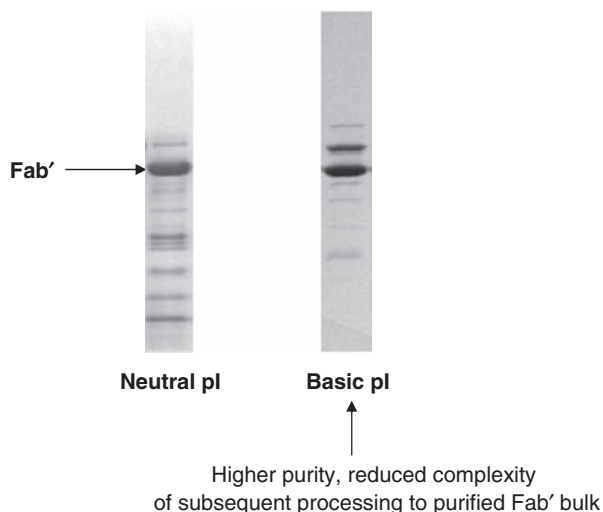
existing antibody–protein interactions (mimotopes), have been identified by screening phage-displayed, combinatorial peptide libraries with an immobilized target protein (30, 31). A synthetic version of the selected peptide can then be immobilized on a suitable matrix. Others have screened combinatorial chemical libraries for antibody mimetic ligands that can also be immobilized on conventional matrices (32). Affinity columns based on chemical ligands are likely to be cheaper and more robust than those based on synthetic peptides, but the peptide display technologies offer greater ligand diversity.

Non-affinity-based chromatography methods are often used as alternatives to Proteins A and G for the purification of antibodies and their fragments. These resins are considerably less expensive than affinity ligands, so there is considerable commercial pressure to avoid affinity capture steps and to reduce purification costs. Resins based on Cibacron Blue dye (33) and thiophilic sorbents (34–37) have been used as a primary capture step. Mixed-mode resins and hydrophobic charge induction chromatography (HCIC) should also be considered for the capture of antibody fragments as they are less expensive alternatives to traditional affinity chromatography.

IEX chromatography exploits differences in charge interactions for the selective adsorption of proteins and is a powerful tool for the capture of Fab' fragments. The isoelectric point of antibodies and their fragments varies widely, from about 4.5 to >9.0, and thus binding and elution conditions need to be determined for each antibody. Fab' fragments tend to be basic, making cation exchange (CEX) a good first step for purification, although conditioning (pH, conductivity) of the feedstream may be required to ensure binding to the charged resin. CEX chromatography has been used to purify a Fab' from microbial feedstreams (Fig. 17.8) (38) and is also a good tool for the efficient clearance of major impurities, in this case endotoxins, DNA, and HCP.



**FIGURE 17.8** Comparison of two *E. coli*-expressed Fab' purifications. **(a)** A two-column process including CEX capture, followed by AEX and HIC, and **(b)** a three-column process including CEX capture and AEX chromatography to obtain purified Fab'. The feedstream at individual process steps was analyzed by Coomassie blue-stained SDS–PAGE run under reducing conditions.



**FIGURE 17.9** SDS-PAGE analysis of an *E. coli* Fab' feedstream following a CEX chromatography capture step, comparing a neutrally charged (pI ~7) humanized Fab' with a basic humanized Fab' (pI ~8). The SDS-PAGE was run under nonreducing conditions, followed by Coomassie blue staining.

Figure 17.9 compares CEX chromatography as the primary capture step for two antibody fragments—a neutral Fab' with a pI of 7 and a basic Fab with a pI of 8. The capture step was optimized in both cases, but higher purity was achieved for the basic product through enhanced separation from HCP, thus reducing the number of subsequent processing steps required. This is demonstrated by SDS-PAGE analysis (Fig. 17.8), which shows that the less-stringent binding requirements for the more basic product led to a cleaner capture pool so that only one additional chromatography step was required to obtain purified Fab'. In contrast, the more stringent binding required for the neutral Fab' species required two downstream chromatography steps to achieve the necessary purity.

The pI of a biopharmaceutical could be genetically engineered to provide the ideal profile required for generic process platforms. This approach reduced the time required for process development when using generic expression systems, enabling preclinical and first-in-human efficacy studies to progress as quickly and safely as possible, as a common process impurity clearance profile can be maintained. Furthermore, the number of processing steps can be optimized by engineering the host cell to change the profile of particular HCPs that tend to coelute with the target product of interest. In one example of this approach (39), the pI of an *E. coli* periplasmic phosphate-binding protein was modified, making it easier to separate from the target protein during the primary capture step, therefore potentially reducing the number of subsequent chromatography steps.



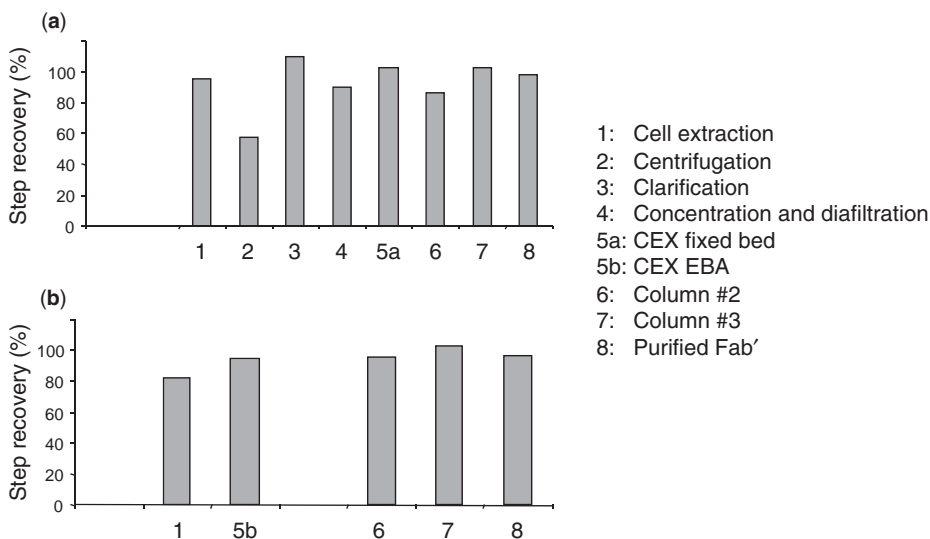
As a general rule, the primary capture step would usually involve step elution rather than complex gradient elution, whatever chromatography format and technique were employed at the manufacturing scale. The product handling, the number of capture pool fractions required for in-process control testing, and the capture pool volume would all be kept to a minimum. Small vessels could therefore be used in the manufacturing plant, reducing the footprint requirements. Product peak collection criteria should be well defined before manufacturing commences, ensuring capture pool batch-to-batch consistency.

### 17.3.3 Expanded-Bed Chromatography

Conventional chromatography columns are operated in a fixed, or packed, bed mode. However, feedstock with a high content of particulates is likely to block a fixed-bed column and must be clarified by centrifugation or filtration (see above). Expanded-bed, or fluidized-bed, adsorption (EBA) offers an alternative mode of chromatography in which nonclarified feedstocks, such as crude fermentation broths, can be applied directly to the column (17, 40–43). EBA resins consist of high-density beads, and by forcing the process stream to flow upwards, the adsorbent expands, facilitating the passage of solids through the column while the product is selectively captured. The EBA resin tends to have a larger diameter than conventional fixed-bed resins, which enables the use of large-pore column adaptor screens. This, together with an optimized distribution plate design, provides an effective primary capture step. The optimal choice of adsorption bead size and density, column hardware, and operating conditions ensure maintenance of bed expansion without loss of adsorbent in the column effluent. The product is typically eluted in packed-bed mode to minimize the volume of elution buffer required. Reducing the number of steps can significantly improve product recovery, and is especially useful for products that may associate with cell debris and therefore may partition with the cells during centrifugation or microfiltration. In these situations, if the affinity of the product for the capture resin can be optimized, then it can dissociate from particulate matter in the unclarified feedstocks. The application of EBA could replace centrifugation, filtration, concentration, and primary capture with a single column step, as shown in Fig. 17.10, where Streamline™ SP CEX technology is used to process a therapeutic Fab' fragment, resulting in significant improvements in product recovery.

Disadvantages of EBA technology include the reduced dynamic binding capacity of the adsorbents and the limited column diameter and tube lengths. It becomes necessary to install a number of columns in parallel, leading to increased buffer usage and also large bed resin volume requirements, or the need to install timely column cycling setups. To address some of these disadvantages, Streamline Direct™ technology has been investigated to assess the capture of antibody fragments from microbial feedstreams (44). This involves a smaller and higher-density resin, giving improved dynamic binding





**FIGURE 17.10** Percentage step recoveries of a manufacturing process comparing recovery of recombinant humanized Fab' using (a) fixed-bed CEX capture and (b) EBA capture process. The latter demonstrates a reduction in the number of unit operations required and an improvement in product recovery.

capacities coupled with salt tolerance, circumventing the need for product stream dilution and therefore reducing loading times and bed volume requirements. Optimization of the column hardware allows flexibility in column tube lengths, reducing the footprint with larger bed volumes. The novel screenless column design ensures potential column blocking by cells and particulate matter is not an issue (<http://www.pabme.com/antibodies>) (52). This improves product recovery, reduces batch cycle times, and can overcome some of the disadvantages associated with EBA technology, making this the method of choice for some industrial applications. However, disadvantages of this system include the high buffer consumption as the column cannot be run in fixed-bed mode, especially during cleaning, sanitization, column conditioning, equilibration, and product elution.

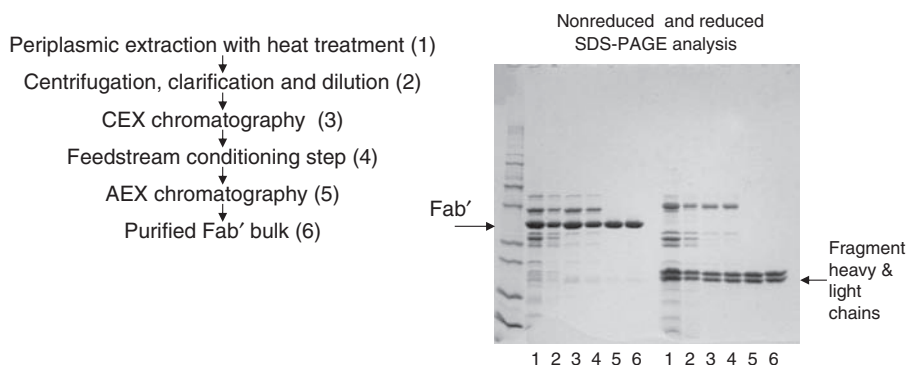
#### 17.3.4 Further Purification and Polishing

Following initial capture, the product stream will need further purification so that the product meets the required quality for preclinical, clinical, and commercial use. This stage of the downstream process often requires a unit operation involving IEX, as the most common impurities are charged species such as DNA and endotoxins (the latter being typical of gram-negative microbial systems). Anion-exchange (AEX) chromatography is used to bind negatively charged impurities, while the product flows through the column. AEX is also used to remove leached ligands such as Protein A from the capture step, and

to remove residual HCP. Column load conditioning prior to IEX often requires a buffer-exchange step (typically tangential flow filtration fitted with appropriate exclusion membranes) to provide optimal product recovery with the correct low-salt buffering environment. However, the increasing availability of salt-tolerant resins means that a buffer-exchange step may not be necessary, simplifying the process and reducing the number of unit operations. Charged membrane technology lends itself to this intermediate purification step. It is a feasible alternative offering the advantages of disposable units with a small footprint and high throughput, shortening time in the production facility and potentially reducing the cost of goods.

Polishing is required so that the pharmaceutical product meets the expected quality control release specification. This step usually requires a high-resolution resin to remove product-related impurities that have thus far coeluted with the product due to common biophysical properties. Typically, impurities removed by polishing include product-related aggregate species, which can be immunogenic, and truncated or deamidated species. Polishing steps can also reduce the levels of process-related impurities such as residual HCP. Polishing steps require chromatography techniques and ligands that are distinct to those used earlier in the manufacturing process.

**17.3.4.1 Intermediate Purification.** AEX chromatography is commonly used in industrial processes as often the second step in purification after either CEX or affinity chromatography. DNA and endotoxins bind strongly to AEX resins facilitating their removal, whereas weakly acidic, neutral, and basic antibodies are eluted. AEX will also remove leached Protein A and many HCP species. If conditions are optimized, the product can be collected in the flow-through fraction, reducing the complexity of the chromatography step and buffer requirements. Residence time should be well defined to ensure residual impurity clearance is robust. The process flow diagram shown in Fig. 17.11



**FIGURE 17.11** Typical process flow for purification of a recombinant *E. coli* Fab', involving periplasmic extraction with heat treatment followed by CEX capture and further purification by AEX chromatography.

demonstrates the use of a two-column step process for a Fab' expressed in microbes, combining CEX and AEX chromatography to yield a product of the required purity.

Advances in the development of salt-tolerant AEX resins has improved processing by removing the need for buffer exchange prior to column loading, which is required for conventional resins. However, a feedstream preconditioning step is recommended to ensure batch-to-batch consistency, especially if the eluate from the previous step can be variable, e.g., in terms of conductivity. Without such conditioning, the AEX step could fail due to insufficient resolution of product and impurities. Strict in-process controls for all column loads should therefore be considered, assessing process operating parameter ranges up front and gaining a good understanding of failure conditions, ensuring that robust parameters are set that can be met and controlled at manufacturing scale.

Conventional AEX and CEX resins can be replaced with membrane technology (see Chapters 14 and 15), which can be considered as disposable options when compared to column chromatography. Membranes have a high throughput and thus a short batch cycle time. Affinity membranes are also being considered as alternatives to column chromatography, as either a capture or product polishing step in industrial processes.

**17.3.4.2 Polishing.** A polishing step can remove residual process impurities such as HCP, and can reduce or control product-related impurities such as aggregates, acidic deamidated species, glycoforms, product isomers, or mispaired antibody chains. Polishing steps to reduce other product-related impurities, such as adducted variants (e.g., glutathione adducts on unpaired cysteines) and truncated products, also need to be considered. HIC separates proteins on the basis of hydrophobicity and can be used downstream of IEX chromatography to achieve highly pure preparations of antibody fragments [as demonstrated in Fig. 17.8 (38)]. Adsorption takes place in a high-salt buffer, and lowering the salt concentration allows progressive desorption. Some antibodies are prone to precipitation and loss of antigen binding in the high-salt environments commonly used during adsorption, e.g., ammonium sulfate. Therefore, HIC may not be suitable for all antibody fragments and must be optimized carefully. High-resolution IEX chromatography resins are also often used as a polishing step—their high resolution coupled with the well-controlled shallow-gradient elution can resolve product impurities otherwise difficult to separate from the parent species. It is important to enforce very tight in-process control measures during polishing, which can be achieved by collecting a number of fractions for rapid in-process control testing. This ensures that product pooling is carried out to maintain the purity of the final product.

Gel filtration (or gel permeation) chromatography can also be used as a final polishing step, even though it is not an adsorptive process. It is particularly useful for the removal of aggregates and degradation products, which are

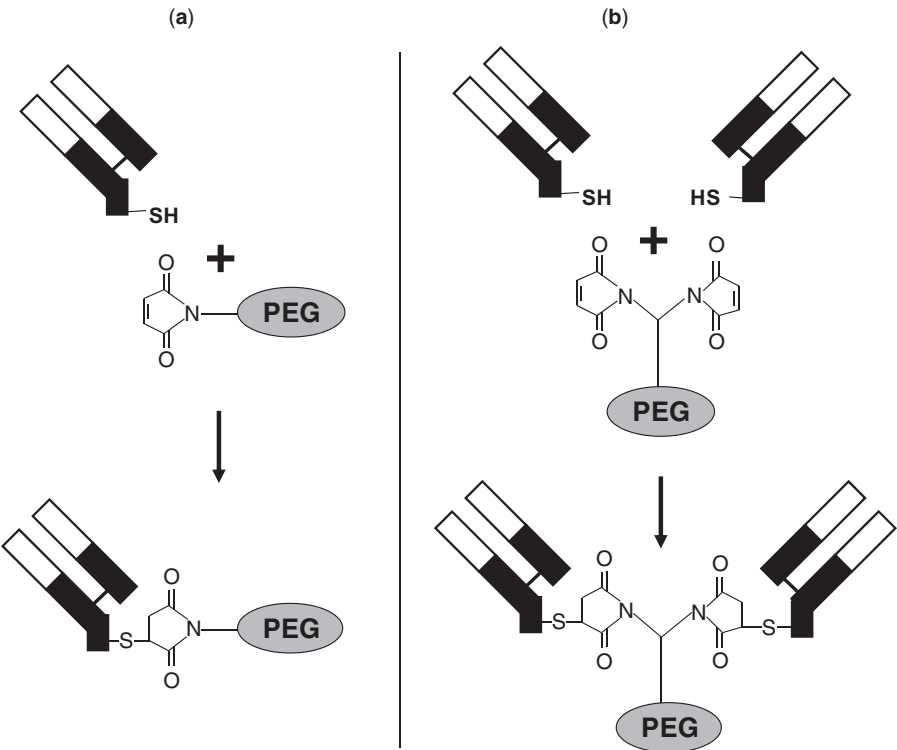
common antibody-related impurities. However, gel filtration is time-consuming and difficult to scale up, and so it is not favored for large-scale processes and is often replaced with more scalable methods such as IEX and HIC (46).

## 17.4 IMPROVING THE PHARMACOLOGICAL CHARACTERISTICS OF ANTIBODY FRAGMENTS

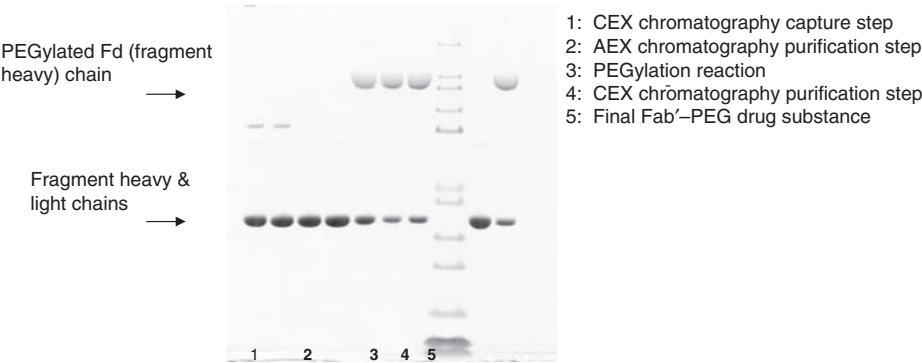
The small size and flexibility of Fab' fragments are often balanced by a reduced half-life compared to the parent mAb (2), so they are usually incompatible with clinical applications where long serum retention is required. However, the short half-lives of Fab' fragments can be overcome by conjugation to a polymer molecule such as PEG (1, 3, 47). Site-specific PEGylation at a suitable distance from the antigen-binding site ensures that the potency and affinity of the V region for antigen binding are retained. Fab' fragments can be linked via a maleimide to PEG at the C<sub>H</sub>1 hinge region, which has been modified to include a single cysteine residue (2). PEGylation at this site has advantages over random PEGylation, which does affect antigen binding (Fig. 17.12a), whereas PEGylation using *bis*-maleimide-reactive PEG can be used to prepare divalent species (Fig. 17.12b). In both monovalent and divalent species, subsequent chromatography is required to remove any nonconjugated Fab' species.

The process flow diagram shown in Fig. 17.13 shows how a Fab' can be purified by IEX chromatography. The Fab' can then be modified by site-directed PEGylation and by subsequent purification using high-performance CEX chromatography, which, when coupled with an effective gradient elution, can be used to control not only by-products (such as nonconjugated Fab' and PEG), but also high-molecular-weight species and acidic species. AEX chromatography has also been used to purify PEGylated proteins (48).

Other methods used to increase the half-life of antibody fragments, e.g., albumin binding to domain antibodies (49), are being considered as viable alternatives in some therapeutic areas. It should also be noted that prior to the advent of recombinant production technologies, Fab and F(ab')<sub>2</sub> were produced by proteolytic digestion of IgG, which is an established technique (17). ReoPro (abciximab), a product used to prevent complications following coronary angioplasty, is a Fab produced by proteolysis of chimeric IgG expressed in mammalian cells. Proteolysis with papain above the disulfide bonds in the hinge region results in Fab fragments that are monovalent for antigen binding. Alternatively, proteolysis below the disulfide bonds with enzymes such as pepsin generates the divalent F(ab')<sub>2</sub> fragment. Digestion with papain also generates the Fc fragment, whereas this is substantially degraded if pepsin is used, and is therefore easier to remove with subsequent processing steps. Purification after proteolysis requires the separation of the desired Fab or F(ab')<sub>2</sub> from undigested IgG, other antibody fragments, and the enzyme used for digestion. This approach is often hindered by scale and is



**FIGURE 17.12** Schematic representation of site-directed PEGylation of Fab' to produce (a) monovalent Fab'-PEG and (b) bivalent di-Fab'-PEG.



**FIGURE 17.13** Manufacture of a Fab'-PEG from *E. coli* expression system, analyzed by reducing SDS-PAGE. The process involves a total of three column steps utilizing IEX chromatography.

time-consuming and complex, so the preferred option is the direct expression of antibody fragments.

## 17.5 CONCLUSIONS

Fab' fragments are exciting new therapeutic modalities in the field of immunotherapy due to their small size, flexibility, and suitability for large-scale production. These therapeutic entities can be manufactured using low-cost microbial manufacturing technology at a scale appropriate for large-market clinical indications. Fab' fragments can be expressed to high levels in *E. coli* and can be chemically linked to other Fab's to confer multiple valency (45). Antibody fragments can also be modified by site-specific PEGylation to increase serum half-life (2). These factors are likely to make antibody fragments the entity of choice for many antibody-based drugs, especially where the effector function of the Fc moiety is undesirable. The low-cost microbial expression system, coupled with low-cost non-affinity-based downstream processing methods, makes this a scalable industrial option that can meet high market demands ensures that patient care is maintained.

Manufacturing costs can represent up to 25% of sales (50, 51), and this has triggered a drive to reduce the use of expensive manufacturing options in order to reduce the cost of goods to within the \$10–\$100 per gram range. The overall cost driver in mAb manufacture is the bioreactor titer, and as high-titer feedstreams and shorter fermentation times become more commonplace, the focus is shifting downstream to eliminate bottlenecks and drive down the cost of goods. Strategies include the use of techniques such as expanded-bed chromatography, which facilitates direct sequestration of product from the fermentation broth, reducing the number of processing steps by combining product capture with some aspects of the primary recovery. Other strategies include the use of synthetic affinity resins or of more rigid adsorbents to enable fast flow rates, which allows faster column cycling and shortens the manufacturing batch cycle. In addition, high-throughput membrane chromatography techniques can be introduced, reducing costs by decreasing cycle times and by implementing disposable systems that avoid expensive and time-consuming reuse studies. In addition, reducing the buffer consumption should always be considered—buffers add greatly to the cost of goods not only because of the cost of buffer components, high-quality water, resourcing buffer preparation, and in-process control testing, but also in installing vessels required for buffer storage and disposal.

Recombinant proteins destined for therapeutic use are made under cGMP conditions to ensure the product consistently meets its predetermined quality attributes. From a fermentation perspective, key objectives include the control of starting raw materials and reproducible fermentation performance in terms of product yield, quality, and HCP profile. The clearance, or avoidance, of fermentation additives must also be considered, e.g., antifoam, antibiotics (used

for plasmid selection), and potential animal-derived components. With higher titers and shorter fermentation times, such systems will drive down costs, making patient care more accessible. The downstream process also needs to ensure consistent yields, batch-to-batch process performance, and intermediate product quality as well as final drug quality. The process should ensure that robust and achievable operating ranges are set, with defined product pooling criteria to maintain in-process product quality rather than release testing alone. Building process analytical technology (PAT) into industrial processing will improve process and product knowledge, allowing real-time assessment and ultimately reducing the need for some end-of-manufacture routine product testing.

Generic processing platforms (see Chapter 13) are valuable tools that can fast-track potential therapeutics from concept and candidate selection through to preclinical safety studies and clinical efficacy studies. As long as the active pharmaceutical ingredient is thoroughly characterized, the process can continue to be optimized so that a commercially suitable process can be installed ahead of larger and more expensive phase III studies. A thorough understanding of the process allows critical control parameters to be established, which is needed to gain a full understanding of the clearance and control of process- and product-related impurities. Appropriate in-process controls should also be introduced to ensure a safe and reproducible process. Following the identification of the critical process parameter ranges, scale-down studies for resin and membrane reuse need to be carried out to support process validation ahead of registering the commercial manufacturing process.

When designing the downstream process for mAb therapeutics, it is important to ensure it is a fully integrated process with upstream fermentation and product expression, primary recovery, and subsequent purification, gaining full insight to any process changes made and the effect these can have on subsequent process steps and product quality. The target product should be subject to detailed characterization early on in development, as not only will this affect the type of downstream process that needs to be designed, but it will also enable process optimization changes to be made at a later stage to ensure a viable commercial process is established and meets regulatory approval. Effective process integration will provide a full understanding of the generation and fate of process- and product-related impurities. It is important to introduce rapid and accurate online in-process control assays, appropriately qualified and validated, to maintain consistent control. Such measures will ensure the high quality of the final product is maintained throughout the various phases of development, through to the industrial commercial process.

## 17.6 ACKNOWLEDGMENTS

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## **PURIFICATION OF ANTIBODIES OTHER THAN IgG: THE CASE OF IgM AND IgA**

CHARLOTTE CABANNE AND XAVIER SANTARELLI

### **18.1 INTRODUCTION**

The production of monoclonal antibodies (mAbs) in the therapeutics and diagnostics industries has risen exponentially over the last few years (1). With the development of Protein A chromatography in the 1970s (2–4), the purification of most immunoglobulin G (IgG) molecules became routine, despite the requirement for one or two downstream polishing steps. Although Protein A is expensive and prone to leaching, it has yet to be replaced by cheaper alternatives such as thiophilic (5) and mixed-mode matrices (6) (see Chapters 4 and 5). Even so, the use of mixed-mode methods such as hydrophobic charge induction chromatography (HCIC) in combination with cation-exchange (CEX) chromatography has been demonstrated for industrial-scale processes (7), as well as CEX and anion-exchange (AEX) chromatography for efficient polishing (8).

Because of the specificity of Protein A for particular IgG subclasses (see Chapter 4), not all mAbs are as simple to capture as IgGs, and different strategies need to be considered for large-scale purification. It is necessary to understand the physical characteristics of the antibody to develop suitable chromatographic matrices (9, 10). The complexity of antibodies predominantly reflects the structure of the heavy chain, which determines the IgG class, the size of the molecules, and glycosylation.

## 18.2 PURIFICATION OF IMMUNOGLOBULIN M (IgM)

### 18.2.1 IgM Structure and Properties

IgM is a 950-kDa multimeric molecule consisting of five 190-kDa units, each comprising two heavy chains ( $\mu$ ) and two light chains ( $\kappa$  or  $\lambda$ ). Each IgM pentamer also includes a single 20-kDa joining chain (J chain) that links the cysteine residues of the heavy chain C-termini by disulfide bonding (11). The glycans present on the IgM molecule can be exploited for purification (12) (see Section 18.2.3.2).

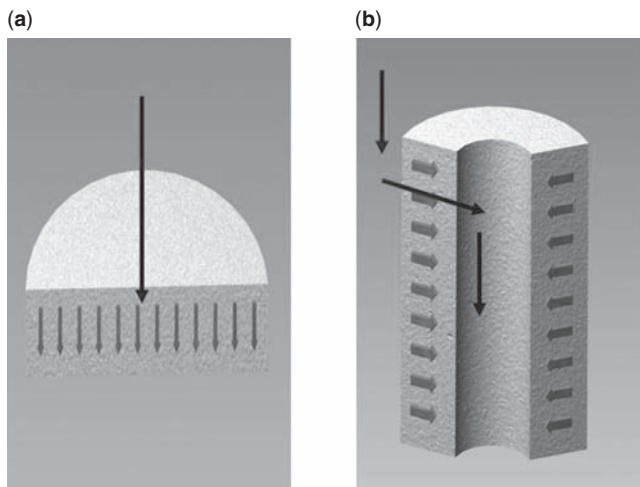
The low stability of IgM molecules and their ability to bind nonspecifically to many materials makes their purification much more challenging than IgGs (13–15). Purification must be carried out rapidly to avoid degradation, and the composition of the chromatography media and buffers must be considered carefully.

### 18.2.2 IgM Purification Technologies

IgM molecules should ideally take the shortest and quickest path through the chromatography column, something that could be achieved using columns with a large cross-sectional area. Although this allows only limited contact with the matrix, the large surface area at the entrance to the column prevents the formation of aggregates, which can form if the product becomes highly concentrated. Radial chromatography meets these requirements and radial flow technology has therefore been adapted for process-scale IgM purification using packed-bed media (16–19), membrane adsorbers (20–32), and monoliths (33–37) (Fig. 18.1).

A comparison of axial technology and radial technology using the same media in a CRIO-MD column (Proxycs Downstream Biosystems, Emmen, the Netherlands) showed that the consumption of equilibration and regeneration buffer at the start and at the end of the chromatographic run was reduced to a single column volume (38). The flow-through and peak volumes were also reduced, reflecting the larger inlet surface area and the small outlet surface area of the column. Flow pathway analysis allowed a technology to be chosen that reduced the total run time and provided a large inlet surface area, to prevent IgM becoming highly concentrated at the column entrance but allowing the product to be concentrated at the outlet.

Media for IgM purification must allow rapid mass transfer, so the choice of bead technology is important. Convective mass transfer must be favored over diffuse mass transfer, so perfusion, hyperdiffusion, and similar technologies must be used instead of classic beads (39). Even so, beads are advantageous because they offer the greatest choice of media and manufacturers. Macroporous beads would seem ideal, since the more porous the gel, the more the effective diffusion of IgM increased (up to  $\sim 7$ -fold greater by reducing the concentration of agarose from 6% to 4%) (40).



**FIGURE 18.1** A comparison of (a) axial and (b) radial chromatography, with black arrows representing the bulk flow of solvent and red arrows showing the flow of solvent across the stationary phase.

Beyond the chromatographic beads, two technologies offer sufficient convective mass transfer to recover IgM quickly—membrane chromatography (41, 42) and monoliths (43) (see Chapter 14). The large pores (1–5  $\mu\text{m}$ ) offer better access for large macromolecules such as IgM and facilitate mass transfer. For example, AEX membranes have been used for the purification of human monoclonal IgM, and the recovery is better than with Superdex and Sephadex (size-exclusion chromatography), Sepharose Fast Flow (AEX chromatography), and maltose binding protein, Protein A, Con Sepharose, and HitrapNHS-Myosin (affinity chromatography). Only a specific anti-IgM matrix performed better. The flow rate had little influence on recovery over a wide range of values.

The advantages of membrane technology are that a high flow rate can be used without back pressure, so large sample volumes can be processed in a short time in mild conditions. Easy scale-up can be performed by increasing the surface area for filtration, which is very interesting for process-scale antibody production. Membranes are also disposable, which reduces or eliminates validation processes. The limitation of membrane chromatography is the capacity, which is lower than the best beads (see Chapter 14).

The advantages of monoliths include their ability to separate IgM from IgG and human serum albumin rapidly and efficiently. After buffer optimization (20mM phosphate buffer, pH 7.2), the authors evaluated the selectivity of different AEX groups—quaternary ammonium (QA), diethylaminoethyl (DEAE), and ethylenediamine (EDA), the last of which gave the best resolution, although with a lower dynamic capacity compared with QA. The results confirm that monoliths are efficient for IgM purification (43).

As is the case with membrane chromatography, monoliths can be used at a high flow rate, and linear scale-up is easy using prepacked columns. The main limitation of monoliths is scaling up to very large column volumes, above several liters. The choice between the different technologies must be guided by the scale of the samples, the type and the concentration of contaminants, and the concentration of IgM.

### 18.2.3 Affinity and Pseudoaffinity Matrices

**18.2.3.1 Protein L.** After Proteins A and G came the development of Protein L, which binds specifically to the variable domain of Ig kappa light chains without interfering with antigen binding (44, 45). Protein L binds to a wider range of Ig classes than Proteins A and G (IgM, IgA, IgE, and IgD), and also binds the single-chain variable fragment (scFv) and fragment antigen binding (Fab) derivatives, but does not bind Igs with lambda light chains.

Because IgM has a pentameric structure that sequesters Fc inside, it does not bind to either Proteins A or G, and Protein L therefore seems an interesting option. It is available from two suppliers as Protein L (Pierce Biotechnology, Rockford, IL, USA) and rProtein L (Actigen Ltd, Cambridge, UK). Actigen Ltd can produce at least 10L, and larger amounts can be produced on request.

**18.2.3.2 Mannose-Binding Protein (MBP).** MBP is a lectin that recognizes mannose residues on glycoproteins. The glycosylation of human IgM thus plays an important role in its purification using this reagent (46). Five N-linked glycosylation sites are present on each  $\mu$  chain [Asn-171, Asn-332, Asn-395 bear complex glycans, whereas Asn-402 and Asn-563 contain essential oligomannose structures to which MBP can bind (12, 47–49)]. Several studies have shown that MBP can be used as an IgM capture reagent (50–55). The interaction is  $\text{Ca}^{2+}$  dependent, so the loading buffer contains  $\text{Ca}^{2+}$  at 4°C, whereas the elution buffer contains the  $\text{Ca}^{2+}$ -chelating agent ethylenediaminetetraacetic acid (EDTA) at room temperature (55).

The use of ImmunoPure immobilized mannan binding protein (Pierce Biotechnology) gave promising results in the laboratory (55), but constraints in terms of regeneration and reuse of the matrix limit its application at the process scale. Leaching and stability during cleaning also need to be investigated.

**18.2.3.3 Thiophilic Matrices.** Thiophilic adsorption chromatography (TAC), developed by Porath and colleagues in the 1980s, was rapidly applied to IgGs (56–58). The first thiophilic gel was generated by reacting divinylsulfone with 2-mercaptoethanol, giving rise to a linear ligand with two sulfur atoms. Adsorption is promoted by highly concentrated sulfates and phosphates, and elution involves reducing the salt concentration. Although the



elution conditions are similar to those used in hydrophobic interaction chromatography (HIC), the interaction mechanism is distinct (57, 59). Hutchens and colleagues (60) have developed a simultaneous purification method for secretory IgM, IgA, and IgG from colostral whey using selective thiophilic adsorption.

The evolution of thiophilic chromatography into heterocyclic thiophilic chromatography began when Porath and Oscarson (61, 62) showed that aromatic or heterocyclic compounds adsorbed antibodies when attached to the matrix with a thioether bond. The 2-mercaptopyridine ligand (HiTrap IgM purification HP, GE Healthcare Life Sciences, Uppsala, Sweden) gave promising results at the laboratory scale with different sources (ascites fluid, serum, cell supernatants) with or without pretreatment (63–67). This product has yet to be used at the process scale, but could be available as a custom design.

**18.2.3.4 Immobilized Metal Affinity Chromatography (IMAC).** IMAC was used successfully to purify an IgM recognizing mutant amidase from *Pseudomonas aeruginosa* (68, 69). The adsorption was due to the histidine residues available in the third constant domain ( $\text{CH}_3$ ) of the IgM heavy chain. The authors tested several stationary phases and metals, including Cu(II), Ni(II), Zn(II), Co (II), and Ca (II). One-step purification was performed on an EPI-60-IDACo (II) agarose column with 98.4% of recovery. The Igs from pig serum (IgG, IgM, and IgA) were purified by IMAC using nickel-iminodiacetate–Sephacrose in a single step (70). However, heavy metal contamination of the feedstream could reduce the application of IMAC in industrial IgM purification trains.

**18.2.3.5 Hydroxyapatite.** Hydroxyapatite has been used as a chromatographic matrix since 1956 (71). Several publications describe the purification of IgM using hydroxyapatite at various places in a multistep process (72–74). One-step purification was achieved from ascites fluid by Stanker and colleagues (75) with good results. The development of ceramic hydroxyapatite (Bio-Rad laboratories, Hercules, CA, USA), which is more useful for process-scale applications, has increased the industry's awareness of hydroxyapatite and its potential (see Chapters 5–7). A recent development is the purification of IgM by coupling hydroxyapatite to monolith AEX chromatography as a polishing step without intermediate concentration or buffer exchange (76).

**18.2.3.6 Protein A Mimetic (TG 19318).** TG 19318 is a tetrameric tripeptide (Arg–Thr–Tyr) synthesized from a central polylysine core connected to glycine. It was selected by screening a multimeric peptide library that was initially synthesized to identify a less-expensive replacement for Protein A (77). Although its selectivity is broader than that of Protein A, its capacity is lower (78). Unlike Protein A, however, it can withstand harsh sanitization conditions without further capacity losses. Experiments are carried out using neutral,



low-salt buffers, and elution is achieved by changing the pH to acid or alkaline conditions as appropriate.

The binding capacity of different matrices using a purified IgM has been evaluated (78). HyperD (Biosepra-Pall, Cergy Saint Christophe, France) gave the best results (8 mg/mL of gel), probably due to its large pores, which facilitated mass transfer. Three IgM sources were tested (ascites fluid, serum, and cell supernatant), and purity and recovery were each in the range 85%–95% with all three sources. Since TG 19318 was developed for IgG purification, a prepurification stage must be carried out with serum to remove IgG, as the ligand is unable to distinguish IgG and IgM.

This ligand can be produced on a large scale at low cost, which is important for process-scale IgM purification. The interaction between the ligand and IgM is strong enough to capture antibodies from a diluted supernatant (10–50 µg/mL). The commercial name of this support is KAPTIV-M (Tecnogen, Piana di Monte Verna, Italy).

## 18.3 PURIFICATION OF IgA

### 18.3.1 IgA Structure and Properties

IgA is primarily responsible for the humoral immune response in the mucosal immune system. To be secreted, IgA must associate with a J chain to form a dimer (sIgA) and also with a secretory component (79–83). IgA is the second most abundant immunoglobulin in human serum and can be present in both monomeric and polymeric forms (84). The importance of IgA in the mucosal immune system (85, 86) has allowed it to be used successfully for passive protection or for therapeutic intervention at mucosal surfaces (87). Several recombinant IgA molecules have been produced in their dimeric form in different host cells, and the purification procedure is tailored to the different platforms (88–93).

### 18.3.2 Affinity and Pseudoaffinity Matrices

**18.3.2.1 Protein L, Thiophilic Matrices, and IMAC.** As is the case for IgM, the multimeric structure of IgA prevents interactions with Proteins A and G, so Protein L has been evaluated as a possible replacement even though it is too scarce and expensive to consider using at the process scale (see Section 18.2.3.1). There have been few publications dealing with the use of thiophilic matrices to purify IgA as part of a multistep process (60, 94, 95) and only one report considering the use of IMAC (70).

**18.3.2.2 Hydroxyapatite.** IgA has been purified from plasma fractions using a multistep process with hydroxyapatite as one of these steps (96), and it can also separate different molecular forms of murine IgA and IgM (72). Hydro-

xyapatite chromatography could therefore be used as a final step to separate IgA variants (monomers, dimers, and polymers) from cell culture supernatants, replacing the more expensive size exclusion chromatograph (SEC) at the process scale (97).

**18.3.2.3 Jacalin Matrix.** Jacalin is a 40-kDa lectin extracted from jackfruit seeds (*Artocarpus integrifolia*). The lectin recognizes D-galactose, so interactions between Jacalin and IgA are dependent on the quality of glycosylation (98, 99). Although jacalin is useful at the laboratory scale, it is inappropriate for process-scale applications because elution requires the addition of excess galactose, and because harsh sanitization procedures could destroy the lectin (95). The leaching of jacalin from the matrix is likely, and since the molecule is pharmacologically active (it is a T-cell mitogen and a B-cell polyclonal activator), the ligand is not recommended for biopharmaceutical production (100).

**18.3.2.4 Protein A Mimetic (TG 19318).** TG 19318 is suitable for the purification of IgA as well as IgM, and could be used with neutral, low-salt buffers for equilibration and binding, and with acidic or basic buffers for elution. The ligand was evaluated for IgA purification starting with the culture supernatant of murine hybridoma cell line ID 150 (101). Optimal conditions for binding were obtained using 100mM sodium phosphate buffer at pH 7.0, whereas elution was performed with 100mM acetic acid and was neutralized immediately. This recovered 80% of the IgA in a single step. IgA was also purified from serum after a pretreatment with Protein A to eliminate IgG.

**18.3.2.5 Streptococcal IgA-Binding Peptide.** Streptococcal IgA-binding peptide (SAP) is a 50-residue synthetic peptide derived from the streptococcal M protein, Sir22 (M22) (102). The authors have shown that this peptide can be used for the single-step affinity purification and specific detection of IgA, with binding in neutral PBS and elution in 0.1 M sodium acetate, pH 4.0. This interesting ligand has yet to be developed as a commercial chromatographic matrix.

**18.3.2.6 ZIgA Ligand.** Combinatorial protein engineering was used to change the specificity of a 58-amino acid domain derived from Protein A (103). This resulted in the recognition of IgA subclasses 1 and 2, as well as sIgA. The ZIgA ligand was tested using *Escherichia coli* total lysate and plasma spiked with IgA, and the antibody was recovered efficiently with high purity. The equilibration, binding, and wash buffers comprised 25 mM Tris-HCl, 200 mM NaCl, 1.25 mM EDTA, and 0.05% Tween 20 (pH 8.0). The antibody was eluted with 0.5 M acetic acid (pH 3.2). Although this ligand is not available commercially, an affibody based on similar principles is being developed by Affibody AB (Bromma, Sweden) for ELISA or for small-scale purification (depletion of IgA from human plasma) at the laboratory scale.

## 18.4 CONCLUSION

At the laboratory scale, there are many possibilities for the efficient purification of IgM and IgA. The choice of strategy depends on the biological environment of the target antibody and its intended use, and this also determines whether a single-step or multistep process is required. Each immunoglobulin has its own characteristics that could prevent interactions with the chosen chromatographic matrix.

At the process scale, the choice is more restricted due to costs, leachables, validation requirements, and supplier constraints. Therefore, simpler technology such as ion exchange (IEX) remains advantageous for very large-scale processes. Radial chromatography uses a short bed height with a large surface at the inlet and a small surface at the outlet, avoiding aggregation on the surface of the gel and concentrating the sample for elution. Monolith or membrane technologies could be used instead, but different chemistries must be used at each step to increase the selectivity of the total process.

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## PURIFICATION OF ANTIBODIES FROM TRANSGENIC PLANTS

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AND SUSAN L. WOODARD

### 19.1 INTRODUCTION

The search for inexpensive production systems that can generate large amounts of recombinant proteins has resulted in the development of transgenic plants as a new technology platform for protein production. Over the last 15 years, the ability of plants to express antibodies and other therapeutic protein products has been amply demonstrated (1–3). Transgenic plant systems have now advanced to the stage where the quality and quantity of the recombinant proteins they produce may make them a viable alternative to fermentation-based production systems. As protein titers from plants continue to increase, the focus is shifting toward downstream process development and opportunities therein to lower production costs.

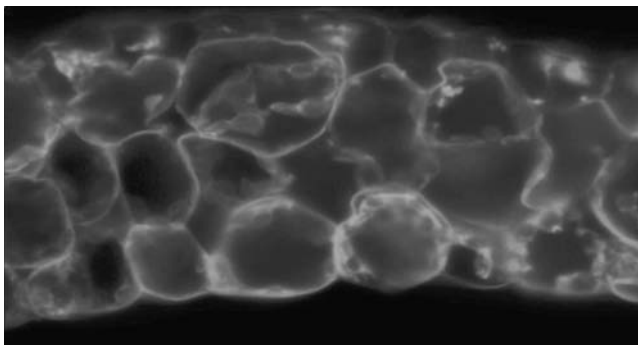
Several process estimates made in the last 10 years show that most of the costs of recombinant protein production in plants are incurred from downstream processing, as is the case in mammalian cell culture systems (4, 5). The potential capital cost savings and the lower cost of upstream production in plants are indisputable, although these savings may be insufficient as yet to encourage the acceptance of this new platform (6). As a result, process development with transgenic plants is currently driven by the need to demonstrate the commercial competitiveness of plant systems. In this chapter, we discuss the advantages and constraints of transgenic plants as a platform technology, particularly with respect to downstream processing.

## 19.2 ANTIBODY PRODUCTION IN TRANSGENIC PLANTS

Monoclonal antibodies (mAbs) have been produced in transgenic tobacco (*Nicotiana tabacum*) (7), moss (*Physcomitrella patens*) (8), alfalfa (*Medicago sativa*) (9), soybean (*Glycine max*) (10), lettuce (*Lactuca sativa*) (11), rice (*Oryza sativa*) (12), potato (*Solanum tuberosum*) (13), maize (*Zea mays*) (14), algae (*Chlamydomonas reinhardtii*) (15), wheat (*Triticum aestivum*) (12), and duckweed (*Lemna minor*) (16). Antibody fragments including single-chain Fvs (17), bispecific Fvs (18, 19), Fv-fragment crystallizable (Fc) (20) and Fab fragments (21) have also been expressed in plants. Transgenic plant systems show a wide variation in mAb expression levels. A comprehensive comparison of the individual expression systems is difficult because no single mAb has been produced in all available systems to provide comparable yield data. However, there is a significant amount of data covering general expression levels in each of the systems, which are usually presented in one of three main ways: as a proportion of fresh weight (FW), as a proportion of dry weight (DW), or as the percentage of total soluble protein (TSP). It is difficult to compare FW and DW values directly because the water content varies in different production systems. In some leafy tissues, water can account for over 90% of the total FW, whereas the water content of seeds is about 10%. Percentage TSP is useful when comparing similar expression systems, but factors such as different extraction conditions and total protein contents must be taken into account when using this comparison. Guy's 13 is one of the best known examples of a plant-derived IgG1, and is expressed at ~1% TSP in tobacco leaves (22). The expression levels attained for some other mAbs in a variety of plant systems are summarized in Table 19.1. A hybrid secretory IgA-G antibody has been expressed in tobacco leaves at 500 mg/kg FW (24). Transgenic moss has produced an IgG4 at 6.5% TSP, equivalent to 5.8 g/kg DW (25). Antibodies have also been produced in transgenic soybean at 65 mg/kg DW (10), in alfalfa at 140 mg/kg DW (26), in lettuce at 80 mg/kg FW (11), and in maize at 0.3% TSP (~30 mg/kg DW) (23). Antibody production at 2.1% TSP has recently been reported in a glycan-optimized duckweed line (27), but other proteins have accumulated to levels as high as 6.0% TSP in this system (equivalent to 1 g/kg FW or 10 g/kg DW). Single-chain Fv fragments have accumulated to 900 µg/kg FW in wheat, 29 mg/kg FW in rice (12), and 30 mg/kg FW in tobacco (28). A Fab fragment has been expressed at 6.5% TSP, equivalent to 510 mg/kg FW, in *Arabidopsis thaliana* (29).

### 19.2.1 Subcellular Localization and Glycosylation

Recombinant antibodies produced in transgenic plants are usually targeted to the secretory pathway. As in mammalian cells, the secretory pathway in plant cells provides an ideal environment for protein folding, disulfide bonding, and subunit assembly into the typical heterotetrameric complex. The endomembrane system of plants is very similar to that of mammals, requiring a signal



**FIGURE 19.1** Confocal micrograph of the immunolocalization of an IgG1 expressed in *Lemna*. An IgG1 antibody expressed in *Lemna* immunolocalized using a goat anti-human IgG1 followed by a fluorescently labeled donkey antigoat antibody. The recombinant antibody was localized in the apoplast of *Lemna* leaves (fronds) as fluorescent blue in all cells of the leaf.

**TABLE 19.1 Comparison of Expression Levels of mAbs and Antibody Fragments in Plant Production Systems**

Production System	Antibody/Fragment Type	Expression Strategy	Expression Level
Tobacco ( <i>N. tabacum</i> )	Hybrid sIgA-G	Leaf	500.0 mg/kg FW
Moss ( <i>P. patens</i> )	IgG4	Secretion	5.8 g/kg DW
Soybean ( <i>G. max</i> )	IgG1	Seed	65.0 mg/kg DW
Alfalfa ( <i>M. sativa</i> )	IgG1	Leaf	140.0 mg/kg DW
Lettuce ( <i>L. sativa</i> )	IgG1	Leaf	80.0 mg/kg FW
Maize ( <i>Z. mays</i> )	sIgA	Seed	~30.0 mg/kg DW <sup>a</sup>
Duckweed ( <i>L. minor</i> )	IgG1	Leaf	1.0 g/kg FW
Wheat plants ( <i>T. aestivum</i> )	Single-chain Fv (scFv)	Leaf	0.9 mg/kg FW
Rice plants ( <i>O. sativa</i> <i>L. indica</i> )	scFv	Leaf	29.0 mg/kg FW
Tobacco ( <i>N. tabacum</i> )	scFv	Leaf	30.0 mg/kg FW
<i>A. thaliana</i>	Fab fragment	Leaf	510.0 mg/kg FW

<sup>a</sup>Reported as 0.3% TSP (23).

peptide for translocation into the endoplasmic reticulum (ER), which contains homologous chaperone proteins, enzymes such as protein disulfide isomerases, and the enzymes of the glycosylation machinery (22). The final default destination for secreted proteins in plants is the apoplast, the soluble phase between the cell wall and the plasma membrane (Fig. 19.1).

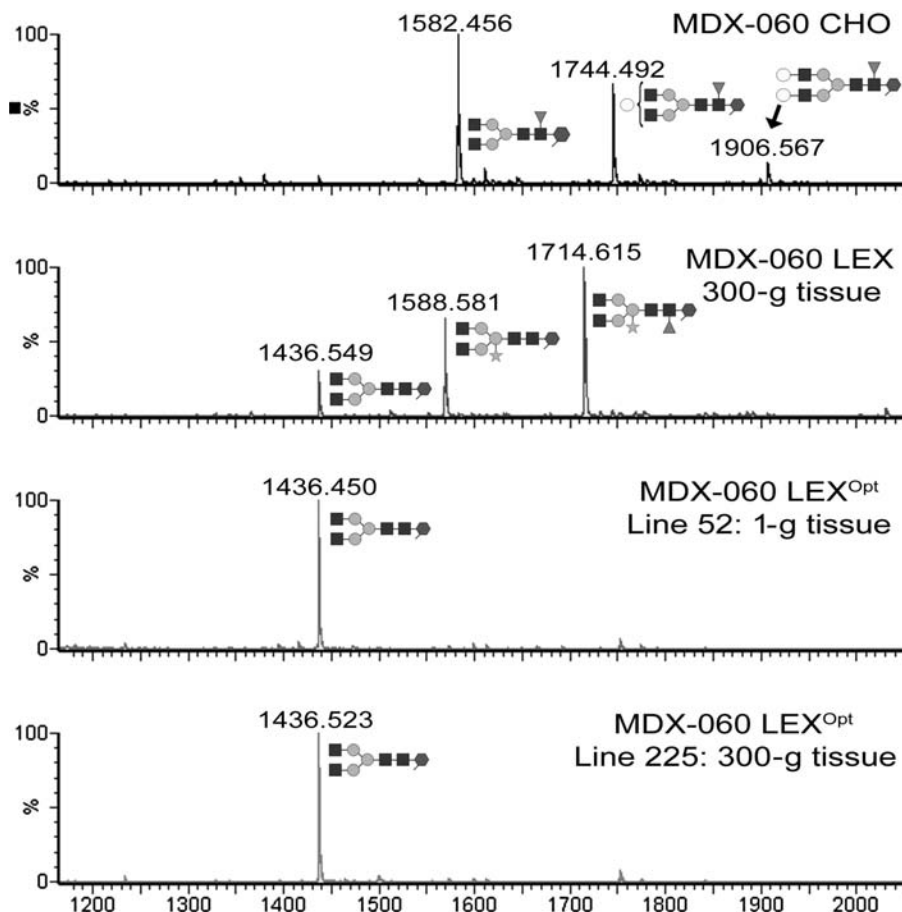
Genetically modified plants can glycosylate recombinant antibodies, adding N-glycans that are similar to those found in mammalian cells (30). Much like animals, plants add predominantly complex-type, biantennary N-glycans. These differ from mammalian N-glycans in that the terminal residue is usually

N-acetylglucosamine rather than galactose. Also, plant complex N-glycans contain  $\beta$ -1,2-linked xylose on the core  $\beta$ -linked mannose residue and  $\alpha$ -1,3-linked fucose (rather than the  $\alpha$ -1,6-linked fucose found in mammals) on the first core N-acetylglucosamine residue. Although there is no direct evidence for immunogenicity caused by plant glycans, it may be desirable to remove them as a precaution, and to enhance antibody-dependent cellular cytotoxicity as this often involves interactions with glycans on the Fc portion of the antibody. The production of antibodies with N-glycans lacking plant fucose and xylose residues has been achieved in *Lemna* (27), moss (31), and partially in tobacco (32). The removal of fucose and xylose in the *Lemna* expression system has also made the antibody more homogeneous, i.e., made up of >95% biantennary N-glycans terminating in N-acetylglucosamine (Fig. 19.2) (27).

Aglycosylated antibodies can also be produced in transgenic plants if their therapeutic activity does not require immune effector function. Aglycosylated antibodies have also been produced in algae (15) and tobacco chloroplasts (33) as well as in several other nuclear transformed plants (34). While aglycosylated antibodies remove the immunogenic risk attributed to plant-associated glycans, there is evidence that aglycosylated mAbs are more sensitive to proteolysis *in vitro* (35), while IgG glycoforms terminating in N-acetylglucosamine are more resistant (36). These data suggest that, in addition to enhancing effector functions, the terminal sugar residues of antibody N-glycans may contribute toward mAb stability by conferring resistance to proteases.

### 19.2.2 Other Factors Affecting mAb Accumulation

Recombinant protein accumulation and stability in plants often varies significantly during the growth cycle and according to growth conditions. The accumulation of a mAb in transgenic tobacco was shown to vary considerably in response to different temperatures and light levels, and as a result of tissue senescence and total protein content (37). Antibody accumulation also appears to be regulated in a developmental context. Top (younger) tobacco leaves, which are the most metabolically active, accumulate greater amounts of mAb compared with middle and bottom leaves (37) (N. Bohorova, pers. comm.) Stevens and colleagues (37) found that the antibody levels correlated with the total amount of extractable protein in various leaves. Proteolytic activity may also be location dependent. Although more overall proteolytic activity was detected in extracts made from upper leaves, the bottom leaves showed more activity on zymograms when loaded on an equal protein basis (S. Woodard and L. Wilken, unpublished data). Consistent with this finding, Stevens and colleagues (37) observed more heavy-chain degradation when purified mAb was incubated in the presence of extracts made from bottom leaves compared with middle and upper leaves. Additional studies have demonstrated *in planta* proteolysis of recombinant IgG in transgenic tobacco through a series of stable intermediates (38–40). This results from stress and tissue senescence when nutrients are remobilized to other plant tissues (37). In seeds, mAb levels may vary because of preferential accumulation of the target protein in endosperm



**FIGURE 19.2** Negative reflectron mode MALDI-TOF mass spectra of the labeled N-glycans. Significant peaks are identified by the corresponding mass ( $[M-H]^-$ ). From reference 27.

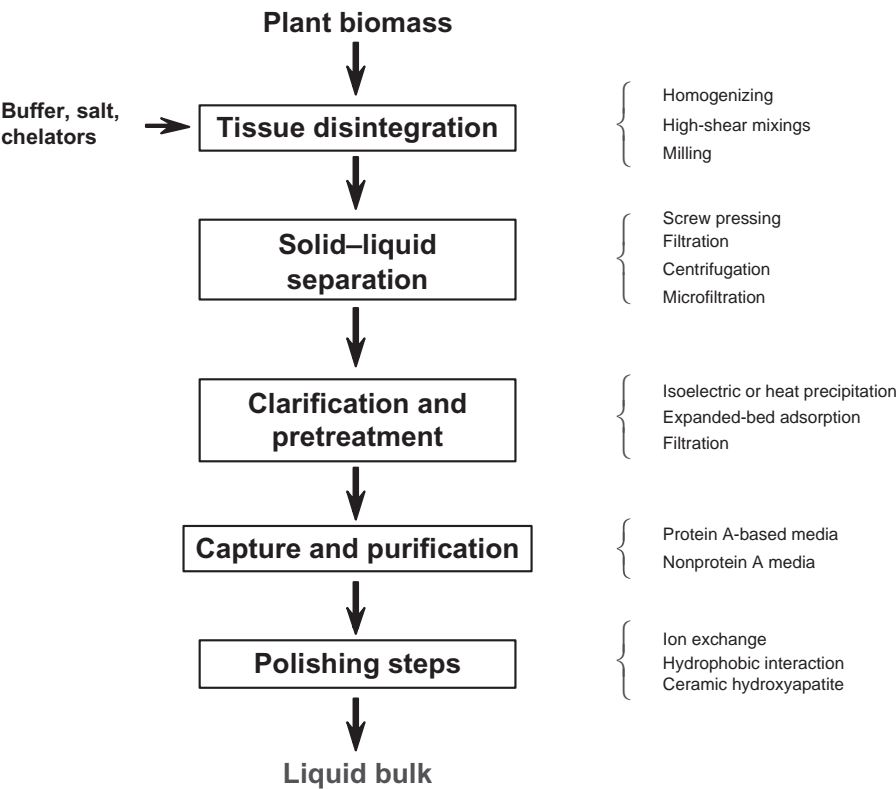
or germ tissue. Whenever prudent, fractionation of transgenic seed to remove low-expressing tissue provides a way to enrich transgenic biomass for the recombinant protein (41). Accumulation of mAbs in plant stems and stalks is substantially lower than in leaves, and the accumulation levels reported in the literature generally exclude stem and stalk tissue.

### 19.3 DOWNSTREAM PROCESSING OF ANTIBODIES PRODUCED IN TRANSGENIC PLANTS

The overall production costs (upstream and downstream) of recombinant proteins is influenced by the total protein content of the biomass, the ease of extraction, the extract complexity, and the stability of mAbs in the harvested

tissue and extract. Seeds are advantageous vehicles for mAb production because of the stability they confer and the relatively high protein content; recombinant proteins can therefore be stored for years prior to processing with no significant impact on the protein quality or yield. The ease of protein extraction from watery tissues (leafy plants) is often seen as a benefit over extraction from dry material, which may require additional unit operations (seed grinding and/or soaking) (3). On the other hand, dry seed extracts have minimal proteolytic activity compared to leaf extracts, in which protein products can suffer rapid degradation (42).

Generic downstream processing steps for transgenic plant tissue are shown in Fig. 19.3. These include (i) disintegration of tissue/cells to release the target mAb, (ii) solid–liquid separation, (iii) pretreatment of crude extract, (iv) product capture and purification by affinity chromatography or other biospecific interactions, and (v) product polishing. The primary product recovery steps include disintegration, solid–liquid separation, and pretreatment. Because



**FIGURE 19.3** Flow diagram for typical steps used in isolating and purifying mAbs expressed in transgenic plant tissue.



Protein A and other biospecific methods typically play a dual role as capture and purification methods for mAbs, they will be discussed in the purification section. An exception is expanded-bed adsorption (EBA) affinity, which is part of the primary recovery process.

### 19.3.1 Tissue Disintegration

The breakdown of plant tissue and the disruption of the cell walls are necessary to maximize the release of the recombinant protein into the extraction buffer. Physical disruption of leafy tissues and seeds can be accomplished by using “off-the-shelf” equipment such as hammer mills for wet and dry grinding (Bepex Rietz, Fitzpatrick), high-shear rotor–stator mixers (Silverson, IKA Werke, Kinetmatica AG, Ross Mixers), and high-pressure homogenizers (Microfluidics, BEE Intl. Inc.) Efficient cutting, disintegration, and/or pulverization of transgenic plant tissue is required to maximize cell disruption and product release. To maintain product quality, process conditions such as buffer volume, shear stress, temperature, pH, ionic strength, and buffer-to-tissue ratio are optimized to strike a balance between yield and quality. Leafy tissues such as alfalfa and tobacco contain a significant amount of fiber from stems and stalks, and these are cut and disintegrated by one- or two-stage hammer milling (43, 44). Macerated (pulped) leaf material is then pressed to produce green juice extract. Other wet physical disintegration scenarios that are currently suitable for seeds and leafy tissues include a combination of hammer milling and high-pressure homogenization or high-shear mixing. Seeds with low oil contents, such as maize and rice, are amenable to a dry pulverization followed by low-shear mixing with an aqueous buffer to extract the target protein (41, 45). In contrast to the processing of aqueous suspensions, the increase in temperature and shear forces during dry grinding and pulverization do not affect product stability. Kinetic studies of recombinant proteins extracted from transgenic plant tissues suggest that shear disintegration and duration are important factors influencing particle size and enhancing the mass transfer of recombinant proteins. A study in which a recombinant protein was extracted from dry-ground canola seed by low-shear mixing in an aqueous solution revealed that slow diffusion of the protein to the surface of the swelled particles governed the extraction kinetics (46). The use of high-shear mixing reduced the extraction time from transgenic canola almost fivefold (Y. Bai and Z. Nikolov, unpublished data). Similarly, the extraction of a mAb from *L. minor* by single-stage, high-shear mixing at 6000 rpm required half the time compared to the same process carried out at 3600 rpm to achieve the same product yield in the extraction buffer (S. Woodard and L. Wilken, unpublished data).

The release of recombinant proteins can be augmented by adjusting the pH and ionic strength of the extraction buffer, and by adding surfactants to reduce molecular interactions between the target protein and cell debris, organelles, and native plant proteins. A variety of extraction conditions have been tested



for the aquatic plant *L. minor* to determine the optimal buffer composition for the release of intact mAb. A buffer containing 0.3 M sodium chloride at pH 7.0 yielded the best results for extraction by high-shear homogenization. The buffer-to-tissue ratio was also investigated over the range 1:1 to 10:1, and 5:1 ratio was optimal. Extractions carried out at pH 4.5 or lower released much less total protein and mAb from the plant biomass, while buffer-to-biomass ratios lower than 5:1 resulted in inefficient cell breakage, and less mAb was released into the buffer.

The most critical and challenging aspect of scaling up tissue disintegration is to control process temperature and to minimize side reactions such as proteolysis and phenol oxidation. A two-stage tissue grinding process or a combination of grinding and high-shear mixing may help to control the extraction temperature better than a single-stage high-shear disintegration step. In either case, a mechanism should be implemented to control the extraction temperature, and the impact of the chosen disintegration method on process costs should not be ignored. Some extraction protocols call for the addition of antioxidants and protease inhibitors during disintegration, but this tactic is unsustainable on a manufacturing scale due to the added reagent costs and the breakage of disulfide bonds by inexpensive chemical antioxidants such as sodium sulfite. Milder procedures for product release include vacuum infiltration (47) and enzymatic digestion of cultured plant cells producing mAbs (48). Although these methods may work on the bench or at the pilot scale, their suitability and economic viability for manufacturing-scale operations have yet to be demonstrated.

### 19.3.2 Solid-Liquid Separation

The choice of a solid-liquid separation method depends on the relative densities of the plant biomass fragments and the liquid, the minimum particle size, the viscosity of the homogenate, and particle compressibility. Centrifugation is a common method for solids removal and/or clarification of plant extracts and homogenates because continuous or semicontinuous processing is feasible; the process is easy to scale up, and it can handle a variety of different extracts. Seed extracts have large differential solid-liquid densities and are therefore clarified easily by decanter centrifugation (low G-force). The presence of insoluble starch in the seed extracts of rice and maize permits the use of depth filters (filter presses) or basket centrifuges. Alfalfa and tobacco homogenates are initially screw pressed to remove fibrous tissue, and the resulting green juice is clarified by disk-stack centrifugation (43, 44). Basket centrifugation (centrifugal filtration) can also be used to remove suspended solids from tobacco homogenates, although it may be necessary to clarify the green juice further by a tubular bowl centrifugation (49). Algae, *L. minor*, and plant cell cultures tend to have low levels of lignin and cellulose (fibrous tissue), and their homogenates may be clarified directly by high-G centrifugation followed by depth filtration.

EBA technology, which allows the clarification, concentration, and purification steps to be combined, has also been tested for the capture of mAbs from complex feedstreams. The use of Streamline Protein A resin (GE Healthcare, Piscataway, NJ) for EBA with transgenic plant extracts has proven challenging because of the complexity of the extracts. Direct adsorption of a mAb from a tobacco leaf homogenate using Streamline Protein A led to rapid column blockage and a decrease in resin lifetime due to column fouling by green juice particles and pigments (49, 50). The use of expanded-bed ion-exchange (IEX) adsorption with seed extracts was also unsuccessful due to the frequent clogging of the inlet flow distributor and the incomplete removal of solids from the expanded bed (51–53).

### 19.3.3 Clarification and Pretreatment of Crude Extracts

The efficient tissue and cell disintegration required for antibody release also releases water-soluble plant cell components such as DNA, chlorophyll pigments, alkaloids, phenolics, soluble cell wall polysaccharides and proteases, all of which must be removed from clarified protein extracts to reduce product losses and to increase purification yields. The amount of phenolic compounds released during extraction varies between different tissue types (leaves, roots, seeds) and different plant species. Leafy plants contain chlorophyll pigments and significantly higher levels of phenolics compared to seeds. Seed extracts lack alkaloids and chlorophyll pigments but may contain phytic acid, triglycerides (oil), and lectins that could interfere with protein purification (54, 55). Several methods have been developed for the pretreatment of plant extracts prior to chromatography to remove unwanted proteins and green pigments that are responsible for resin fouling and the loss of resin capacity due to nonspecific binding. The removal of compounds responsible for resin fouling is critical to reduce overall downstream processing costs associated with the purification of antibodies, antibody fragments, and Fc fusion proteins from transgenic plants. Aqueous two-phase extraction has been used for the pretreatment of clarified tobacco extracts prior to Protein A chromatography (56). This fractionation method increased the purity of an anti-HIV mAb three- to fourfold while removing plant phenolics and toxic alkaloids from the Protein A feedstream.

Many mammalian production systems use low-pH hold steps to inactivate viruses in unprocessed bulk or following Protein A affinity chromatography (see Chapter 8). Such steps require lengthy incubations at very low pH to ensure complete virus inactivation. While plant systems do not require viral inactivation (57), the use of a low-pH precipitation is helpful as an extract pretreatment step to remove significant amounts of contaminating host cell protein (HCP) and chlorophyll pigments from the tissue extracts. The selection of correct pretreatment conditions such as pH, ionic strength, and temperature is critical as significant losses of mAb due to precipitation and proteolysis may occur below pH 5.0 (37). Phenolics and green pigments can also be removed

by adsorption to hydrophobic uncharged or anionic resins, but no significant difference has been observed in mAb purity or yield compared to low-pH pretreatment of *Lemna* extracts.

As indicated above, plant proteases are present in homogenates and clarified extracts and are currently dealt with similarly to proteases in other protein production systems, i.e., by reducing contact time and/or temperature, selecting the most appropriate extraction pH, removing known proteases by capture chromatography, and adding inhibitors such as EDTA. An alternative strategy for the protection of plant-derived mAbs both *in planta* and in the crude and clarified homogenates is the coexpression of protease inhibitors. The coexpressed inhibitor may protect target protein in two ways, first by reducing *in planta* degradation and second by reducing proteolytic activity in the crude extracts. Coexpression of the Bowman–Birk serine protease inhibitor with an IgG4 reduced antibody degradation and increased the overall yield of IgG in the biomass (58). Coexpression of cathepsin D and aprotinin in transgenic tobacco also reduced overall degradation of recombinant protein in the tissue extract (59). Identification of the class of protease responsible for recombinant protein degradation is essential if a protease inhibitor strategy is chosen. Other potential strategies include mutation or RNAi knock-down of endogenous protease genes, although their successful application has yet to be reported, perhaps because proteases are important in normal plant development, so inhibiting host proteases may detrimentally affect plant health.

#### **19.4 PURIFICATION OF PLANT-DERIVED mAbs USING PROTEIN A**

mAbs and Fc fusions form the largest category of biopharmaceutical product currently manufactured using mammalian cells, and the same trend is unfolding with plants. In mammalian systems, Protein A affinity chromatography has become the industry-wide workhorse for the capture and purification of mAbs (see Chapter 4), and as would be expected, the use of Protein A has extended to plant systems. Protein A capture takes advantage of the interaction between the Fc portion of antibodies, antibody fragments, or Fc fusion proteins and the Protein A ligand through hydrophobic and hydrogen-bonding interactions at neutral pH (60). Adjustment of the resin to a lower pH is sufficient to disrupt these interactions and elute the protein. The pH required for elution is protein dependent, and the optimization of wash and elution pH (intermediate pH values) can be used as a means to remove nonspecific proteins and to limit mAb aggregation. The use of Protein A resin for the capture of mAbs and Fc fusion proteins provides a reliable and robust single chromatography column process capable of achieving high purity. The downside of the process is the high cost of the resin and the limited number of cycles that can be carried out with each batch of resin. Specifically, chlorophyll pigments and phenolics in

crude extracts are known to foul chromatography resins, leading to a significant reduction in the resin lifetime.

In addition to the classic binding site, some mAbs interact with Protein A resins through an interaction with the variable domain (Fab region) of the antibody heavy chain (61–64). Proteolytic activity in transgenic plants can produce Fab and Fab'2-like fragments (40) which may interact with traditional Protein A resins through the variable region (especially the VH<sub>3</sub> subfamily of antibodies). The similarities between such fragments and the intact mAb make separation of the species by traditional means difficult. A new type of Protein A resin, SuRe (GE Healthcare), designed to withstand the alkaline conditions used for resin regeneration, has a lower affinity for the variable regions of the VH<sub>3</sub> subfamily and allows less-acidic conditions to be used for the elution of many antibodies (60). As a result, mAb aggregation problems associated with low-pH elution can be reduced (60). The cleanability of SuRe Protein A resin has increased its lifetime and the number of times it can be recycled. The total number of cycles that can be achieved is still being evaluated. The use of inexpensive anion-exchange (AEX) resins like Dowex 1 × 2 in flow-through mode provides an option for removing those components of tissue homogenates responsible for resin fouling.

## 19.5 PURIFICATION OF PLANT-DERIVED mAbs USING NONPROTEIN A MEDIA

The high cost of Protein A resin for the capture of mAbs and Fc fusion proteins has led to the evaluation of alternative process schemes for the purification of mAbs from both mammalian cell cultures and plant tissue extracts (see Chapters 5–7). The ability to use traditional chromatography resins for protein purification varies according to the complexity of the feedstream. Three-column process trains consisting of Protein A, IEX, and hydrophobic interaction chromatography (HIC) have often been used for the manufacture of mAbs produced in mammalian systems (65). The limitations of traditional IEX and HIC media for the purification of mAbs from plant extracts have not been thoroughly evaluated, and occasional attempts have not met required expectations. Cation-exchange (CEX) resins work on the charge interaction principle, so the antibody affinity can be manipulated by varying the solution pH. Typically, mAb binding to CEX occurs at or below pH 5.0 and acidic proteases, found in plant systems, can degrade antibodies in acidic environments (37). This degradation can be reduced at low temperatures, but the long process times required for commercial manufacturing would almost certainly reduce mAb stability in acidic buffers. The degradation products are often difficult to remove because of their similarity to the full-length molecule, and the losses that are incurred during subsequent removal steps impact on the overall process yield. Aggregation induced by low pH may also be a problem with CEX resins, especially when the feedstream pH is at or below pH 4.5.

Immobilized metal affinity chromatography (IMAC) binds intact IgGs and can remove some proteolytic fragments from the feedstream (66), and Blue Sepharose resin has also been shown to bind mAb under neutral conditions. The downside of both resins is the low mAb capacity (<18 g/L) compared to the traditional Protein A resins (>30 g/L). Alternative capture resins designed with mAbs specifically in mind include hydrophobic charge induction chromatography (HCIC) resin and Protein A mimetic ligands. HCIC is based on the pH-dependent behavior of heterocyclic ligands that ionize at low pH values. These resins could capture mAbs efficiently and could achieve purities ranging from 70% to >99% as determined by SDS-PAGE (67, 68). Protein A mimetic ligands (MAbsorbent A1P and A2P) are small molecules designed to mimic the Protein A–Fc interaction. Such ligands have been immobilized on agarose resins and can thus tolerate harsher regeneration conditions (69). MAbsorbent resins have been shown to work quite well with mAbs produced in mammalian cells, but they perform poorly with plant extracts. In summary, alternatives to Protein A capture have not met the demands and expectations of the mammalian cell culture platform and will not perform any better with plant tissue extracts, which typically have higher HCP content than mammalian cell culture broths.

Two additional naturally occurring IgG-binding proteins, Protein G and Protein L, have been used for antibody capture. Protein G binds the same region on the Fc portion of the mAb or Fc fusion as Protein A, but has broader subclass specificity. The specificity of the resin is useful in a research setting, but the differentiation of subclasses is typically not required during commercial manufacturing. There are several limitations for this resin including the harsh elution conditions required, the lower binding capacity, and infrequent use on a commercial scale. Protein L binds to the kappa light chain of the Fab portion of mAbs and mAb fragments, including single-chain Fvs (scFvs). Like Protein G, Protein L is also not used in any current commercial manufacturing facility, and the binding capacity of the resin is significantly lower than that of Protein A (70–72).

## 19.6 POLISHING STEPS

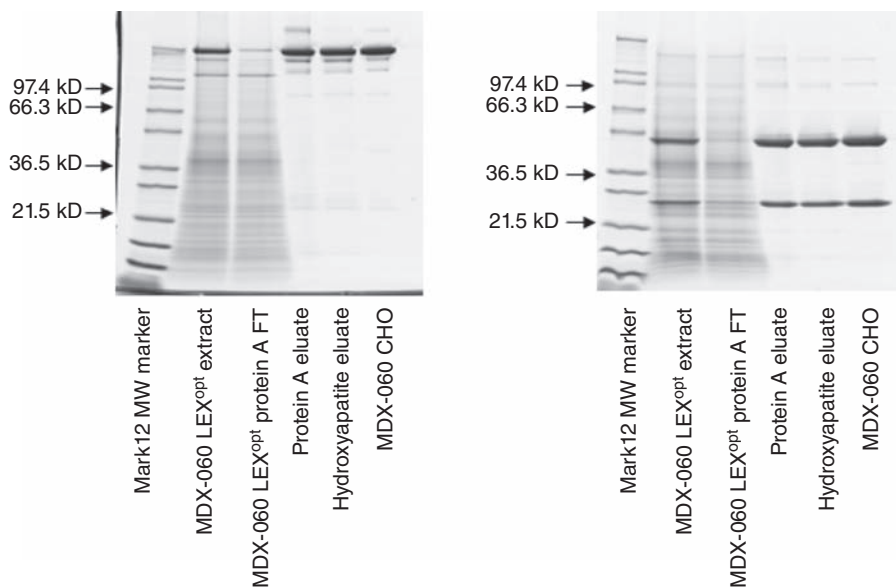
One or two polishing steps are usually required following Protein A chromatography to remove minor HCP contaminants and antibody-related impurities (see Chapter 7). Purification from plant systems differs from mammalian systems in that no viral inactivation steps are required. Plant viruses are not known to infect humans, and human viruses do not replicate in plant cells (57). As a result, low-pH hold steps that can reduce mAb quality and yield are not required when using plant production systems. Antibody-specific impurities, such as antibody fragments, misassembled antibody species, and mAb aggregates, must still be removed from the Protein A-captured material. In addition, HCP, DNA, endotoxins, and leached Protein A must be removed regardless of

the production system. Leached Protein A is known to be immunogenic in humans and has been proven toxic in many clinical studies (73). General purification schemes for the removal of aggregate and host proteins typically involve the use of CEX chromatography following the Protein A capture. Different charges on the mAb aggregates and the intact heterotetramer allow separation using a simple NaCl gradient. In some cases, the low pH required for binding to the CEX column can reduce the protein quality. CEX also fails to clear HCP in many cases. HIC is frequently used for polishing as it can remove aggregates from the intact product (74, 75). HIC resins are known for their low capacity and require high salt concentrations to induce mAb binding. The addition of salt to the Protein A eluate and the relatively high salt concentration of the HIC eluate increase overall process time and cost in large-scale manufacturing. HCIC resins have recently become more popular as a polishing step after Protein A chromatography. The ability of HCIC resins to bind mAbs at relatively low salt concentrations has facilitated the transition from Protein A elution to polishing chromatography by eliminating the requirement for large-scale feedstream manipulation (75). AEX resins and membrane anion exchangers are useful in flow-through mode for the removal of endotoxins, DNA, and HCP. AEX chromatography is often used as a final step in the production process to remove all remaining impurities prior to final formulation (76) (see Chapter 5). Ceramic hydroxyapatite (CHT, Bio-Rad, Hercules, CA) has been used as a polishing step because of its ability to remove endotoxins, DNA, leached Protein A, and aggregates (74). CHT Type I resin was used to purify a fully human MDX-060 antibody from *L. minor* and was able to remove all aggregated forms of the antibody, as shown in Fig. 19.4 (27).

The use of CHT in commercial manufacturing has been limited because column packing is difficult in large-scale processes; the resin has a short lifetime, and the buffer conditions that maintain resin stability without degradation of the calcium phosphate backbone are very restrictive. Recent improvements in resin design have removed most of these drawbacks.

## 19.7 CONCLUSIONS

Downstream processing strategies for mAbs produced in plants have mimicked the general state of the art that evolved for mammalian cells. Protein A has become the workhorse for capturing and purifying mAbs from cell culture, and improvements in affinity chromatography media have catered to this platform. As this chapter points out, the main differences between cell culture media and leafy plant extracts containing mAbs are the presence of chlorophyll pigments and phenolics in the latter and the absence of mammalian viruses. The oft-cited advantages of Protein A as a mAb capture step for mammalian cell cultures include the significant reduction in virus titers and the high degree of purity that can be achieved. Because the presence of phenolics and pigments may seriously diminish the reusability of Protein A



**FIGURE 19.4** SDS-PAGE of plant extracts and protein A or hydroxyapatite-purified samples from MDX-060 LEX<sup>opt</sup> under nonreducing and reducing conditions, respectively. MAb purified from a CHO cell line (MDX-060 CHO) was used as a positive control. Note that MDX-060 LEX<sup>opt</sup> protein A FT is the nonbinding portion or flow-through. Mark12 molecular weight (MW) markers were included on the gels. Gels were stained with Colloidal Blue. From reference 27.

media, it has become necessary to steer away from Protein A as a capture step. In addition, plant extracts do not carry and propagate mammalian viruses, so the virus reduction achieved by Protein A-based media is not such an advantage when processing plant extracts. If pretreatment of leafy extracts becomes necessary to increase Protein A resin lifetime, one should consider developing alternative capture methods. It appears prudent at this point of plant platform development to focus process research efforts on designing efficient pretreatment methods to reduce the concentration of impurities such as HCP, pigments, phenolics, and proteases. Once effective pretreatment methods are established, less-expensive purification trains can be designed by reducing the number of subsequent chromatography steps, thus fulfilling the economic objectives of using plants in the first place. Future platform development objectives should include reducing the level of critical impurities *in planta*, controlling their release during cell disintegration, the analysis of plant homogenates, and the establishment of process analytical methods. These objectives, if addressed at this early stage of plant platform development, will allow the implementation of quality-by-design concepts, which will be easier to introduce *de novo* compared with changing established manufacturing practices.



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## ANTIBODY PURIFICATION: DRIVERS OF CHANGE

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### 20.1 INTRODUCTION

Antibody purification will have a robust future based on the strong pipeline of products in development and the wealth of diverse strategies for purification, as discussed in the preceding chapters. These strategies will continue to be refined, and in some cases replaced, as technologies are developed offering superior performance, or the same performance with lower costs. That being said, what factors are likely to drive continued change? At the highest level, the eternal business challenge is to continue to do more with less. For antibody purification, this means continuously shortening development times while simultaneously gathering more and more data to support process understanding and to improve process economics, all with increasing resource constraints and zero tolerance toward compromises on safety. Economic pressures are increasing at a time when public perception of the biopharmaceutical industry is lower in confidence with regard to safety, and cynical about costs. The largest biotechnology companies increasingly resemble large pharmaceutical companies as they face the inevitable problems, associated with scale, of slowing growth rates and increased competition. In this environment, many companies are forced to make process development decisions earlier in the product life cycle, and as such are making more risk-averse decisions with respect to technology implementation.

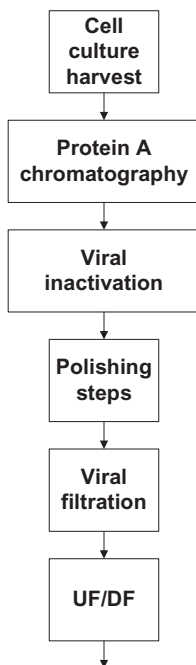
Upstream development has responded heroically, and titers as high as 10g/L have been reported (1), although lower titers may be preferred if

they can be achieved in shorter time frames. Increasing titer reduces material costs and increases equipment and facility utilization upstream, but increases the relative costs downstream (see Chapter 12). From a purification perspective, costs are driven by the mass of material to be purified, and as titers increase, so must the scale of columns (and to a lesser extent filters) to keep pace.

The current strategy used to commercialize monoclonal antibodies (mAbs) is to develop a robust platform process that can be applied as broadly as possible to different candidate molecules in order to avoid spending time refining details when the most important priority is to test the candidate in the clinic (2, 3). Ideally, once the efficacy of the molecule has been established, the commercial process can be developed and characterized. However, since the major drivers are speed and efficient use of resources, in many cases the approach has been to continue to scale up the platform approach (on which the clinical data is based) rather than to develop a truly cost-effective commercial process. Many companies are also choosing to mitigate risks by postponing optimization until the potential for commercialization has been assured, and then submitting post-approval modifications. The area of post-approval process changes will be discussed later in this chapter.

Most mAb processes today are based on the large-scale use of Protein A (Fig. 20.1; see Chapter 4). Scaling up these processes to cope with larger quantities has clearly been shown to be technically feasible (4), and indeed sensitivity analyses have shown that there are opportunities for significant savings in material costs. However, purification remains the most significant cost and is dominated by the cost of Protein A resins (5), which over the lifetime of a product may amount to hundreds of millions of dollars. Non-affinity-based antibody processes are discussed earlier in the book (see Chapters 5 and 6), and so from a technical and regulatory perspective, eliminating Protein A is perfectly feasible. However, most companies choose to continue with the expensive Protein A resin, since it is a very efficient and reliable step in the process, achieving high product quality and yield, and removing host cell protein (HCP) and viruses. If Protein A is not used, additional steps (resins or filters) are often needed in the process to achieve the same product quality. At the end of the day, many manufacturers will say that additional steps (equating to cleaning of equipment, capital, labor, etc.) are more costly than the cost of the Protein A resin. In that situation, many manufacturers will argue that the merits of Protein A outweigh the costs.

Innovations in process technology will also have a significant impact on current and future facilities in terms of the fit compared to current processes, and the relative allocation of space required for purification and associated buffers and cleaning solutions. Facility costs remain the most significant production costs, and any improvements in productivity and facility utilization have a significant benefit, assuming they can be captured without excessive disruption.



**FIGURE 20.1** Platform downstream process for mAbs. UF/DF, ultrafiltration/diafiltration.

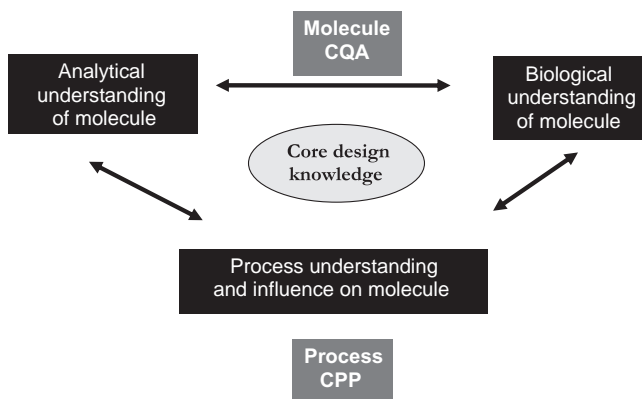
Technical alternatives to the standard platform approach to antibody purification are certainly emerging, but the reluctance of manufacturers to adopt them is a reflection of the conservatism in our industry. This conservatism is driven by two factors—the regulatory burden of making changes and whether a new technology/approach fits within existing facility designs. Few manufacturers will embrace a technology if it makes their current facilities obsolete, or if the technology is for a single process and is not applicable across multiple products. The process economics have to be very compelling to embrace change. This conservatism has led to an incremental approach to process improvements and technology adoption. Along with continued improvements in the current technology, this conservatism will remain a barrier to innovation for some years to come. Some of the technical alternatives have been reviewed recently (6, 7), so rather than reiterate and reassess these alternatives, we shall instead focus on some of the topics that influence change and that may facilitate the stepwise incorporation of improvements. These include, but are not limited to, the changing regulatory environment, coupled with the ever-increasing ability (and requirement) to generate data, the emergence of innovative analytical and control technology, process economics, the globalization of our industry, and finally, follow-on biologics (FOBs).



## 20.2 THE CHANGING REGULATORY ENVIRONMENT— PHARMACEUTICAL MANUFACTURING FOR THE TWENTY-FIRST CENTURY

During the first part of this decade, the Food and Drug Administration (FDA) set out to reshape the way the industry approached manufacturing science and technology. In particular, the industry was seen as wasteful and inefficient, and quality was perceived as being achieved through test and rejection rather than from well-designed and well-controlled processes. A recent study supports this view (8). This has led to Pharmaceutical cGMPs for the 21st Century, an initiative for product quality regulation that utilizes a science- and risk-based approach, thus facilitating innovation. The elements of this approach are based on improvements in process design, where quality is designed into the process from the outset [quality by design (QbD)]. This is based on a thorough scientific understanding of the relationship between the product and the process by which it is made. Critical product quality attributes (CQAs) are monitored during the process, either directly or through surrogate markers, and critical process parameters (CPPs) are controlled through a responsive process to reduce variability and to provide greater quality assurance (Fig. 20.2). The aim is to encourage manufacturers to innovate and implement the most recent scientific advances into manufacturing processes by reducing the regulatory burden, based on increased understanding that the manufacturer has gathered throughout the life cycle of the product from development through to commercial manufacture. Shorter cycle times, less waste, improved automation and reduced human error, and real-time product release are all benefits that could be gained.

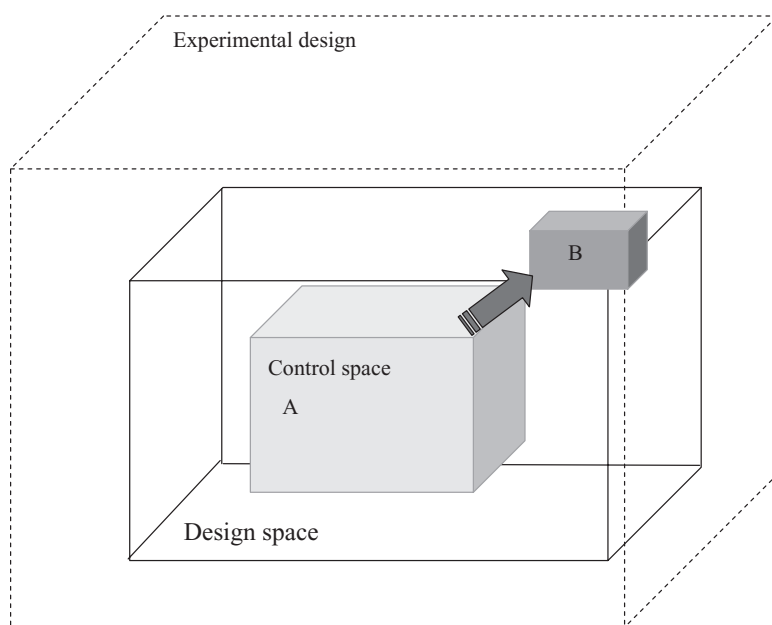
Implementing change in pharmaceutical processes, even as an improvement, has been seen as difficult, and often requires clinical data to permit



**FIGURE 20.2** QbD is based on an understanding of the relationship between the product and the process by which it is made.

implementation. QbD introduces the concept of design space, which describes the relationship between process inputs in terms of variables and process parameters, and product CQAs. A systematic approach to process characterization is required, which begins with risk assessment and prioritization, process modeling (at the laboratory scale) and characterization, and the setting of acceptance criteria (9, 10). Theoretical approaches are useful for modeling chromatographic steps (11, 12). Also, experience from earlier, similar processes should be leveraged. Development of process design space takes place after the decision to commercialize a molecule, during process characterization, since there is little point in performing extensive experimentation before efficacy is established.

Once an acceptable design space (within which satisfactory product quality is achieved) has been established, a manufacturer should then have flexibility to modify the process and to move within that design space with less regulatory burden (Fig. 20.3). This provides significant benefits to the manufacturer,



**FIGURE 20.3** Relationship between design space and control space. Experimental design tests a range of conditions to establish which of those conditions are able to produce a product of acceptable quality on a consistent basis. This is the design space. A manufacturer will normally operate in a range of conditions within the design space to give additional confidence of acceptable quality. This is the control space. If, however, a manufacturer is able to implement improvements that allow operation within a different control space (B) to the original (A), the manufacturer can implement the changes and move within the design space with a minimal regulatory burden. The new space may occupy a zone of higher yield or lower cost or variability.

permitting greater freedom to make improvements to the process and facilitating the transfer of processes from one site to another and even from one manufacturer to another, provided the appropriate analytical tools and assays are available.

Exactly how these initiatives will be implemented is still the topic of debate. In addition to scientific literature, a framework of practices, standards, and guidelines is being worked on by professional societies and regulators. The International Conference of Harmonization (ICH) develops guidelines for quality, safety, efficacy, and multidisciplinary topics. Section Q8, Pharmaceutical Development, describes the concept of design space, whereas Section Q9 provides guidelines for risk management. International standards organizations such as the American Society of Mechanical Engineers (ASME) and American Society for Testing and Materials (ASTM) International are developing consensus standards for implementation approaches, and professional societies such as the Parenteral Drug Association (PDA) and the International Society for Pharmaceutical Engineering (ISPE) are developing technical reports, guides, and white papers for specific operations. These documents provide an important framework for the more detailed interpretation of ICH Guidelines, and consensus standards in particular give users a level of confidence in how practices will be accepted by regulatory authorities (13).

Combining QbD with process analytical technology (PAT) allows for the development of a dynamic process that can respond to input variability. It follows that such a process should be able to respond to changes better and to facilitate incorporation of improvements in process technology, be they changes in equipment or even in critical processing aids such as resins and filters (6).

### **20.2.1 Using Design Space to Enable Change**

A typical antibody purification process consists of centrifugation and filtration to remove particulates and to concentrate the product, followed by a series of chromatography steps to further concentrate the antibody and to remove contaminants and impurities. There are also specific steps to inactivate and remove viruses (2, 3, 14). Each unit operation can be broken down further to materials, equipment, operating conditions, and control strategy. In each case, the extent to which a step can be modified or even eliminated will depend on the impact on the process and product quality. Materials include process water, chemicals and buffers used in processing, and filters and resins, as well as other ancillary product contact materials such as tubing, bags, columns, skid components, and valves.

In-line monitoring of total organic carbon (TOC) can be used as continuous assurance of water quality. Chemicals and buffers used during processing are generally simple molecules and can be qualified on the basis of supplier specifications. However, attention must be paid to the source, because even simple salts from different suppliers can vary noticeably in conductivity at the same

concentration (15), with a consequent impact on process performance and product quality.

Nonproduct contact filters, such as vent and buffer filters, serve to protect unit operations from particulates and microbial contamination and have no direct impact on the process itself. As such, there is relatively little risk attached to switching between one and the next, provided satisfactory performance and durability can be established.

Filters with product contact, like resins, can have considerable impact on process performance and product quality, and here the approach to change is more conservative, increasingly so the closer one gets to the end of the process. For example, the risk posed by a change of filters used in primary product clarification is not as significant as the risk posed by a change of filters used for virus filtration. The differences are chiefly in the materials and design, which offer differences in critical performance properties such as flux and capacity. Their ability to perform in a given unit operation may depend to some extent on the preceding steps, so performance should be verified. Consideration should also be given to the methods and materials used in moldings, fittings, and seals as potential sources of leachables and extractables. It is their proximity to the final product that contributes most of the risk. Because of the platform nature of antibody processes, the user can also use data developed with newer filters and processes to support the change in older processes.

The challenging aspect of making process changes lies with the very specific resins and membranes used in the process (see Chapter 2). Although many of these modalities are available from multiple suppliers using similar or even identical chemistries, they are not necessarily interchangeable. The differences arise from differences in resin chemistry, surface area, particle size, and porosity, and result in differences in retention, elution conditions, product and impurity elution profiles, and choice of cleaning conditions (it is worth noting that there can be similar variability between different lots of the same resin from the same manufacturer and this should be explored when selecting suppliers). As a result, changes to resins, or even the removal of a particular resin step, are seen as the most significant changes that can be made to a process.

Despite these concerns, examples exist where changes to resins and purification steps have been made to approved processes. Historically, Protein A was manufactured using plasma-derived IgG. Suppliers have addressed concerns that this may be a potential source of virus contamination by switching to conventional purification methods and by showing that the Protein A was equivalent (16). The resin was used in a commercial process and the users demonstrated that the antibody was also equivalent to the licensed material produced by nonclinical means (17). Furthermore, in this example, due to additional improvements to the purification process, a hydrophobic interaction chromatography (HIC) step was found to be redundant and was subsequently removed from the process (18), serving as a further example of change.

Another possible opportunity for change is to substitute an ion-exchange (IEX) column, whose function is to reduce virus contamination, with a

membrane absorber of similar charge. There may need to be some fine tuning of the process, but it should be possible to demonstrate equivalent performance by nonclinical means (see Chapter 14).

Demonstration of product equivalence with a modified process should be straightforward using the full battery of analytical methods and assays that manufacturers have at their disposal. This includes virus and DNA clearance, the presence of aggregates, and the removal of HCP. The remaining doubts would center on whether the profile of residual HCP and/or aggregates would be sufficiently different from the original process—or indeed any other antibody preparation produced by similar means—to result in adverse reactions. This should always be discussed in advance with regulators and an appropriate submission strategy developed.

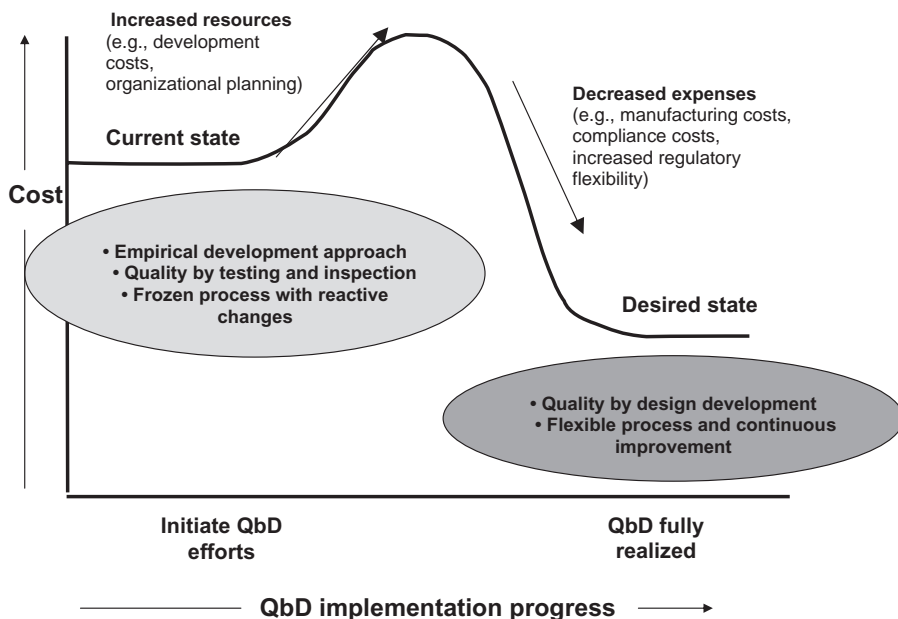
Improvement in equipment design may be the least daunting of reasons to consider when making a change to a process, since the performance of the unit operation can be enhanced without changing the scientific basis for the separation. Improvements are often designed to reduce the dead or hold-up volumes in equipment, which can cause dilution in samples, back mixing, and product losses. Cleanability may also be improved. Filter housings, valves, columns, and skids are all candidates, and integrated designs are becoming more commonplace. More significantly, changes could be incorporated to allow for a more advanced control strategy, such as the implementation of PAT.

In all cases, equipment and component materials must be chosen on the basis of fitness for purpose, including cleaning and potential sources of leachables and extractables, if there is contact with the product or process streams. Equipment design will vary, most noticeably with scale, and it is important to have reliable models capable of predicting performance as one undergoes a transition from one scale to the next.

The main barrier to the introduction of improved equipment designs has been the time and cost required for validation. Fortunately, new approaches to equipment validation are emerging and of particular significance is the new ASTM Standard E2500, which offers a modern, risk-based approach to verification (19). This approach streamlines repetitive testing, such as installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ), reduces non-value-added documentation, redefines the role of quality and the use of supplier/manufacturer expertise, and lowers the barriers to implementing change (20).

Adopting such practices and other regulatory practices under discussion, notably for continuous verification (see ASTM website, E55 committee), may simplify the adoption of more complex operating modes, such as simulated moving beds, and may facilitate continuous processing.

In the future, it will be possible to design and operate processes with a considerably higher degree of understanding and consequent flexibility than in the past. This will facilitate improvements in unit operations and technology transfers from one scale to the next or from one facility to the next, with differences in equipment, but only when a significant amount of data has been



**FIGURE 20.4** Costs and benefits of QbD implementation. Adapted from Nasr, M.A. 2006 PhARMA API Workshop, CDER, FDA.

gathered to support the change. This may require more data during characterization and a heavier investment in the early stages of development, but the reward will be greater flexibility and lower costs after commercialization (Fig. 20.4).

### 20.2.2 High-Throughput and Microscale Approaches to Process Development and Characterization

An obvious hurdle to a well-defined design space is the need for an impractically large number of experiments, a direct consequence of the large number of variables (e.g., materials, process conditions) contributing to any given process step. This requirement to do more with less is also germane to process development in general, where the need is to develop as many molecules as one possibly can (more shots on goal) with limited resources. The availability of automated liquid handling tools heretofore used for high-throughput screening has led to their adaptation for process purposes in a paradigm now referred to as “bioprocess on a deck” (21). This offers a very convenient method for running a large number of experiments at the microscale with limited material and personnel requirements. Although the greatest challenge is to obtain comparable scale-down models at the microscale level, this approach provides a very useful relativistic tool. Microbioreactor systems are already under evaluation for upstream processes (22–24), as are high-throughput tools for cell line development (25). In purification, chromatography

development using microtiter plates has been widely demonstrated for proteins (26–28), including mAbs (29). The concept has been extended further to a new chromatography format (30) and envisioned for the entire bioprocess (31). This area is expected to grow as more tools are developed to show relevance at the large scale.

Microscale experimentation has recently been demonstrated in the development of a process for a recombinant vaccine for human papilloma virus (29). Here the authors introduce a new variant of microscale chromatography where the resin is actually packed in pipette tips used in the automated liquid handler. The authors compare the three different methods of experimentation—a conventional laboratory-scale column, a microwell batch adsorption format in 96-well plates, and the micropipette column format in an eight-channel pipettor. The two microscale formats have different advantages and disadvantages, but both approaches provide a 10- to 15-fold greater experimental throughput compared with conventional laboratory-scale column chromatography while using at least a 10-fold smaller sample volume. As well as reducing time per experiment, the labor required per experiment is also significantly reduced with these microscale approaches. Further development and adaptation of these kinds of tools will be necessary to exploit their full potential.

## **20.3 TECHNOLOGY DRIVERS—ADVANCES AND INNOVATIONS**

Emerging technologies driving change are not confined to alternative separation methods leading to alternative unit operations. Rather, these technologies impact the way in which unit operations are run or the way in which facilities are designed and resources deployed. Two areas that show signs of significant impact are the emergence of new analytical and data handling tools, which in turn impact control strategies, and the use of disposable technologies.

### **20.3.1 PAT**

New analytical and data handling tools offer significant opportunities for improvements to existing processes with relatively little disruption. Sir Humphrey Davy (1778–1829) wrote that “Nothing tends so much to the advancement of knowledge as the application of a new instrument. The native intellectual powers of men in different times are not so much the causes of the different success of their labors, as the peculiar nature of the means and artificial resources in their possession.”

New tools give us new insights into process performance and product quality. The FDA Guidance for PAT (32) has grouped these into four areas:

1. multivariate tools for design, data acquisition, and analysis;
2. process analyzers;

3. process control tools;
4. continuous improvement and knowledge management tools.

Multivariate data analysis (MVDA) is gaining popularity as a tool for gleaning more information from the extensive data sets we already have by applying statistical tools such as principal component analysis (PCA) and partial least squares (PLS) to study the multidimensional nature and the interdependence of multiple material attributes and process parameters (33). These tools can be readily applied to upstream applications where a multitude of input and process parameters can be studied, such as pH, temperature, conductivity, mixing, pO<sub>2</sub>, pCO<sub>2</sub>, and ion concentration (34). In purification, correlation between material attributes (e.g., resin particle size, surface area, porosity, capacity), process parameters (e.g., column bed height, sample load, residence time, buffer pH, conductivity, gradient slope), and outputs (e.g., retention time, peak volume, peak shape, transition analysis) can lead to the development of models that give a better indication of packing integrity and process performance (35), data that can better support the replacement of one resin with another as described above. Care should be taken with MVDA approaches to establish a causative relationship between observed trends and the mechanistic understanding of the process, and although they are attractive for evaluation of upstream processes because of their complexity, they may find more immediate usefulness in the downstream area, where they can be applied to preexisting theoretical models.

The standard process analyzer in a purification processes is ultraviolet (UV), used to detect and track protein products. Although ubiquitous, UV is not able to distinguish between the product and related contaminants. Near infrared (NIR) is widely used in small-molecule processes, and it is immediately applicable to test the identity of materials. The need for rapid analytical tools for the detailed analysis of products and product-related impurities (e.g., aggregates, misfolded variants, and glycosylation variants) has been proposed for at least 20 years (36), but little progress has been made in terms of deployment. Online high-performance liquid chromatography (HPLC) has been used to determine peak quality (37) and to monitor sample loading on to Protein A columns (38), but the problem lies in shortening analysis times to seconds rather than minutes. Similar limitations are faced by mass spectrometry and capillary electrophoresis (39), and one solution is to use HPLC analysis to develop predictive models for feed-forward control either prior to sample loading (40) or during fraction analysis (41).

### 20.3.2 Process Control Technology

Software developers have recognized the need for improved process control and knowledge management tools. These tools allow the development of sophisticated models for process control and facilitate data retrieval, and in combination with new analyzers can lead to a high degree of assurance of



process performance and therefore confidence in product quality. These innovations can reduce waste and improve efficiencies, and in some cases even allow for greater freedom in implementing process changes. The ultimate innovation required in this area would be the ability to extrapolate from physicochemical structure to biological properties, such as efficacy and immunogenicity. There is speculation that biosensor technology may help in this respect (42).

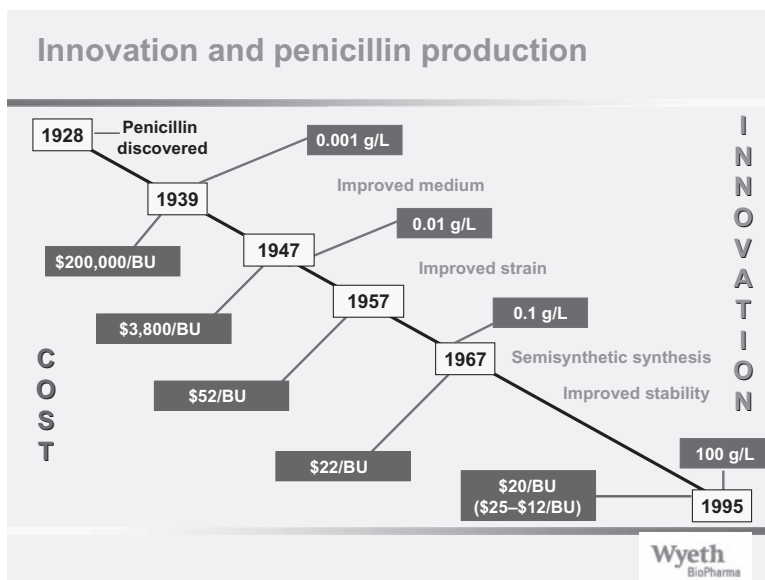
## **20.4 ECONOMIC DRIVERS**

### **20.4.1 Cost of Goods (COG)**

The economics of antibody manufacture has recently been reviewed (5) (see Chapter 12). As stated previously, various components make the manufacture of antibodies expensive—from the cost of Protein A resin to the high cost of capital facilities, they all contribute unfavorably to the cost picture. Reducing the COG is always a manufacturing goal. At the purification end, approaches have included resin and membrane reuse, replacing anion-exchange (AEX) chromatography with a membrane equivalent, reducing the number of chromatography steps to two, and influencing the choice of antibody molecule to forms less prone to undesirable processing characteristics such as aggregation. Approaches to replace packed-bed chromatography with membrane separations have been demonstrated as a proof of concept but have not really been adapted to the industrial scale. However, with the cost center being shifted downstream, there is not a more prominent place to look for overall COG reduction than purification. A compelling case for continued improvement in COG has been made by Wyeth (43) by comparing the evolution of the cost of penicillin (Fig. 20.5). In addition to doing more with less during development, this serves as one of the clearest drivers for change.

### **20.4.2 Single-Use Disposable Components**

There is rapid expansion in the use of disposable components in the biopharmaceutical industry. There are multiple drivers for this, including convenience, avoidance of cleaning (and cleaning validation) costs, capital avoidance, and reduction of changeover time (44–48). Their use varies with scale and development stage, and is particularly relevant in the earlier stages of development. As with other components, the materials used and their potential for interaction with processes and the final product need to be understood. Considerable experience has been gathered with many of these materials over time, and there is significant data about their safety characteristics from the various suppliers. Bags may be used to contain product or process fluids, and may be dedicated to specific pieces of equipment (bioreactors, freezers). One problem that is emerging is that the suppliers of disposable components are consolidat-



**FIGURE 20.5** Impact of innovation on cost of penicillin production. From *Penicillin: A Paradigm for Biotechnology*, R.I. Mateles (ed.), Candida Corp., 1998. BU = billion units.

ing with filter suppliers (e.g., Millipore, Sartorius) and instrumentation suppliers (e.g., Thermo, GE Healthcare), and whereas previously the user made choices of bag, filter, and tubing combinations independently, suppliers will clearly have their preferred combinations. Suppliers are also developing increasingly integrated, and therefore unique, assemblies such that there is a risk from single or sole source availability. There is a need for standardization in this area so that components can remain reasonably interchangeable.

### 20.4.3 Globalization

Another driver of change to consider in the realm of the “future of antibody purification” is the impact of globalization on antibody development and manufacture. All of the approved products to date have come from either the USA or Western Europe. However, the success of antibody therapeutics has spawned activity in other regions, partly in the novel biotherapeutics arena, but mostly in anticipation of patents expiring on currently approved antibodies. If small-molecule active pharmaceutical ingredient (API) production is any measure, one can imagine a future increase in antibody manufacturing activity in Asia, among other areas. China and India are now the world’s top two manufacturers of APIs (49), and India has the distinction of having the largest number of drug master file (DMF) submissions and the largest number of US FDA plants in the world (50). The world of vaccines offers another view

of biomanufacturing activity, where the Serum Institute of India sells over a billion doses of vaccines globally, comparable to the largest western vaccine players. In the case of mAb manufacture, Celltrion in South Korea already has FDA-approved large-scale antibody production capability; Lonza and Genentech are building capability in Singapore, and other players are gearing up. It must be self-evident, however, that antibody manufacturing is a different beast compared to API manufacturing or even microbial protein manufacturing (51–53).

Technologies for antibody purification in the emerging areas are initially likely to be no different than in the USA/Europe. However, in the long term, the economics in those areas are likely to demand further innovations to bring down the COG. In Asia, recombinant insulin is already marketed at an 80% price discount, and an antiepidermal growth factor receptor (EGFR) mAb is marketed at a 65% discount (54). It is likely these costs are primarily related to low labor costs, which can further extend into upstream opportunities provided by backward (or vertical) integration. However, as labor costs continue to increase, the need for low costs will demand alternative technologies and, more realistically, alternative practices for antibody purification. Albeit with different extents, the driver of lower cost converges across the different regions. Comparison has been made to the semiconductor industry (55, 56), where manufacturing started out locally as a highly specialized and expensive activity, but over the years has been transformed into a highly distributed, somewhat generic activity with many low-cost players.

Another aspect of globalization could well be the emergence of more decentralized manufacturing, by way of either the entire supply chain or a portion of the supply chain (e.g., filling). In the former case, local plants could manufacture the same antibody under a manufacturing license from a parent, under conditions that are suitable for the local environment. Here, one could envision a greater use of disposables since the overall scale of manufacture could be reduced to a level more suitable for their deployment. This model of decentralization is already underway, driven more by marketing considerations than by manufacturing. An example of the latter case of decentralization is GSK vaccines, where bulk is produced in a centralized location in Europe and then distributed to various parts of the world for final fill/finish. Unlike vaccines, however, the number of doses of antibodies is substantially lower, which would influence the choice of the preferred model.

#### **20.4.4 Follow-on Biologics (FOBs) or Biosimilars**

It is widely believed that FOBs will become an important feature in the industry within the next few years. The FOBs market in Europe and in the USA could be as high as \$16.1 billion by 2011 (57). Many innovator companies agree that while FOBs should be considered for approval, their approval should be based on indication-specific clinical trials. With many of the bestselling drugs coming off patent and with many antibodies not far behind, there are those

who believe that the regulatory agencies will be faced with submissions from other companies wanting approval for FOB versions of these products almost immediately. In fact, with the European Medicines Agency (EMA) approval in 2006 of a “biosimilar” version of human growth hormone in Europe (Sandoz’s Omnitrope), many would argue that the age of FOBs is already here. There are many competitive strategies that can be adopted by the innovating companies, including second-generation molecules with convenient delivery options, pricing strategies, and the development of more complex products. One competitive strategy that the innovator companies can use is to improve their legal protection by improving their manufacturing processes. This strategy works in two ways—it allows the innovators to introduce new technologies into their processes, which have the potential to reduce the COG or to improve the reliability of the process, while also strengthening the patent protection on the process. The innovator can therefore use the comparability process to introduce novel technologies into manufacturing. In addition, the advances in analytical technology referred to earlier in this chapter will also enable the innovator companies to develop a greater depth of knowledge of their own molecules, increasing their confidence in comparability exercises, while potentially making it more challenging for FOBs.

## 20.5 CONCLUSIONS

As we peer into the future, it seems that the drivers of change in the world of antibody manufacturing are not those we might have predicted 10 years ago. As the biotechnology industry has matured, it has also become somewhat conservative like other industries and, in many cases, has adopted an attitude of “if it isn’t broken, why fix it?” Millions of dollars spent on fixed capital costs such as facilities and stainless steel have made many companies reluctant to make significant changes to the three-chromatography-column process first introduced in the 1990s. While expression levels have risen, many groups have shown that it is feasible to recover those large bulks within the constraints of the current processes and structures (4, 58), thereby reducing the need to introduce any innovative technologies to the purification process. Therefore, we are seeing little more than incremental improvements in downstream processing. The technologies are available, but when will anyone really commit to implementing them, when the barriers are so high?

In this chapter, we have identified some critical drivers for changing the way antibodies are produced. What is apparent is that the primary drivers are not the availability or maturity of new technologies, nor the increased bulk sizes, but maybe the regulatory environment, the competition from FOBs, the globalization of the industry, and the consumer pressure to reduce drug costs (COG). With the introduction of the concepts of QbD and design space, the regulatory agencies are requiring more data for each product to be included in the submission. The upside is a smoother path for demonstrating

comparability and lower requirements for product release. However, to support this increase in data collection, there is a requirement for more innovative analytical tools.

For FOBs, the innovator has an opportunity to introduce new technologies in the development of second-generation products, either in the manufacturing process or as part of the drug delivery mechanism. This serves two purposes—to compete with any FOBs by providing a competitive, convenient product and also by tightening the patent protection around the product from a manufacturing perspective. The clarification of the process to demonstrate comparability when process changes are made enables an innovator to introduce new technologies, knowing that there is a path to approval for these second-generation processes.

Ultimately, the continued evolution of antibody purification will be a function of the continued popularity of antibodies as a therapeutic modality. Technologies such as directed molecular evolution, which optimizes the primary amino acid sequence (59, 60), or other approaches targeted at glycosylation (61) are capable of improving specificity and avidity, substantially lowering dosages, and consequently the need for very large-scale purification of antibodies. The use of fragments instead of full-length antibodies (62) would have an enormous impact on purification strategy, certainly displacing the use of Protein A (although it would remain useful for fusion proteins where a receptor target is coupled to the Fc region to improve stability or to recruit an immune response). Even further in the future is the potential to replace antibodies with synthetic entities such as avimers, highly specific molecules based on multiple epitopes derived from human extracellular receptor domains (63). These molecules are extremely stable and of relatively low molecular weight, so they represent a very different (and simpler) purification challenge.

Perhaps the major therapeutic revolution of the latter part of the twentieth century was the emergence of antibiotics, one manifestation of Ehrlich's "magic bullet." Whether antibodies or their descendants will occupy a similar preeminence in the twenty-first century will depend on whether developments in antibody processing can keep pace with, or even outpace, the disruptive technologies mentioned in the paragraph above. This will be an intriguing question for our descendants to resolve.

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